Nuclear Import Pathway of the Cytokine Interleukin-5 and its Receptor Subunits

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July 2002

A Thesis Submitted for the Degree of
Doctor of Philosophy
of
The Australian National University

Nuclear Signalling Laboratory
Division of Biochemistry and Molecular Biology
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Except where otherwise acknowledged,
this thesis represents my own original research

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ACKNOWLEDGEMENTS

The work on this project has been a long and challenging process. I am pleased to have finished this project in due time, but I am also mindful of the huge debt that I owe to many people who gave freely of their time, expertise and friendship over the course of my PhD.

My time at the John Curtin School of Medical Research at the Australian National University was the most intellectually stimulating time of my academic career. The ANU gave me a generous scholarship and ample travel funds for attending national as well as international scientific conferences, for which I am most grateful.

I have been particularly fortunate with my advisory panel: David Jans, Ian Young, David Tremethick, Frances Shannon and Paul Foster. David Jans has been an inspiring supervisor, from whose profound knowledge in the area of Nuclear Signalling I greatly benefited. I am most thankful for his continual encouragement to take on the challenge of this anything-but-mainstream research project, for his great enthusiasm and his never-ending optimism and support throughout the duration of my PhD. But equally important for a successful time "down under" was his devotion to unveiling the deeper secrets of cricket to me!

Ian Young generously shared his immense knowledge of the biology of cytokines, and of IL-5 in particular, and provided me with expert advice whenever needed. All of my panel were helpful and friendly, and balanced this with trenchant criticism when needed.

Special thanks to my fellow PhD students, Sophina Calanni, Jade Forwood, Chong-Yun Xiao, Chee-Kai Chan and Melanie Johnson-Saliba for creating such a stimulating working environment as well as for making this multicultural Nuclear Signalling Lab work so beautifully. I will always cherish the great social interactions both in and out the lab. Lyndall Briggs, Patricia Jans, Anna John, Lakshmi Wijeyewickrema, Assad Jadeer and Chenoa Barton are thanked for providing such a friendly working environment, but most

importantly, for doing such fantastic cell culture work. Thanks to Mark Lam for providing truncated forms of importin β.

My thanks go out to the members of Ian Young's Cytokine Signalling lab: Sally Ford, Sonja Gustin, Alice Church and David Mann. I am particularly grateful to David for introducing me into the world of Molecular Biology, to Sally for her assistance with the cell culture work and Alice for doing such a wonderful job on the protein purification line.

Many thanks to the members of the gene-expression groups, especially to Adele Holloway and Sudha Rao, for their assistance with fractionation and immunoprecipitation procedures. This work was also greatly assisted by the members of the EM, FACS and Biofacility units: Sabine Grüninger, Geoff Osborne and Peter Milburn.

I would like to record my great debts of gratitude to my Graduate House friends: Richard Dennis, Yuki Tanaka, Steve Jarvis, Paul D'Arcy and Xiaoqin Emma Fan for their invaluable friendship, but especially for always reminding me that there is a world outside the lab. In particular, I am most grateful to Paul and Xiaoqin for their support throughout the process of writing this thesis.

For their unwavering support and generosity over the years, special thanks to my parents Monika and Kurt Jülich as well as my sister Dörthe, who I wish a successful time on her own PhD project at Yale. But to one person I give deeper and more heartfelt gratitude than to all the rest put together: my partner, Shizue Matsubara.

Dedicated to the memory of my grandmother.

ABBREVIATIONS

bp	Base pairs
BSA	Bovine serum albumine
BPB	Bromo phenol blue
cDNA	Complementary DNA
CLSM	Confocal laser scanning microscopy
CWFG	Cold water fish gelatine
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EM	Electron microscopy
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FDC	Factor-dependent continuous cell line
FGF	Fibroblast growth factor
Fn/c	Ratio of nuclear-cytoplasmic fluorescence
G-CSF	Granulocyte colony-stimulating factor
GH	Growth hormone
GHBP	Growth hormone binding protein
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GST	Glutathione S-transferase
HRP	Horseradish peroxidase
HSA	Human serum albumine
HTC	Hepatoma tissue cell line
IFN	Interferon
IB	Intracellular buffer
IP	Immunoprecipitation
IPTG	Isopropyl-β-thiogalactopyranoside
JAK	Janus kinase

JNK	Jun N-terminal kinase
kb	Kilo bases
kDa	Kilo Dalton
LB	Luria Bertani
LIF	Leukemia inhibitory factor
MAPK	Mitogen-activated protein kinase
MCS	Multiple cloning site
MWCO	Molecular weight cut off
NE	Nuclear envelope
NGF	Nerve growth factor
NLS	Nuclear localisation signal
NPC	Nuclear pore complex
NTS	Nuclear targeting signal`
Nup	Nucleoporin
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PKC	Protein kinase C
PRL	Prolactin
PTHrP	Parathyroid hormone-related protein
RPMI	Roswell Park Memorial Institute
SDS PAGE	Sodium Dodecyl Sulfate Poly-Acrylamide Gel electrophoresis
Sfx	Fluorescein-X succinimidyl ester
SH-2	Src homology 2
STAT	Signal transducer and activators of transcription
TAE	Tris acetic acid + Ethylenediamine tetra acetic acid buffer
T-ag	Tumor antigen
TNF	Tumor necrosis factor
TRF	T cell-replacing factor
WGA	Wheat germ agglutin
X-Gal	5-bromo-4-chloro-3-indoly-b-D-galactoside

ABSTRACT

Interleukin 5 (IL-5) is an eosinophil differentiation and activating factor and, as a consequence has a central role in regulating eosinophilia associated with allergic disease and parasitic infections. The biological activities of IL-5 with respect to eosinophils are well established, and many intracellular IL-5 receptor-mediated signalling pathways have been elucidated. However, these pathways are not exclusively activated by IL-5, but are shared by IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF), which, together with IL-5 utilize a common signaling entity, the β_c-receptor subunit.

A functional bipartite nuclear localization signal (NLS) has been identified in IL-5, implying that nuclear localization may constitute a novel, ligand-specific pathway through which the distinct effects of IL-5 in eosinophil activation are achieved (Jans *et al.,* 1997ab).

The present study represents the first elucidation of the detailed mechanisms of nuclear localization of IL-5 and its receptor subunits. *In vitro* nuclear transport assays were applied to shed light on the mechanisms of IL-5 nuclear transport, whilst the nuclear translocation of both IL-5 and its receptor were verified in the context of a whole living cell, employing biochemical techniques including cell fractionation, protein precipitation and Western blotting.

Examination of the IL-5 sequence of both mouse and human revealed that a third short cluster of basic amino acid residues ("NLS3") is present between the formerly identified basic clusters of the bipartite NLS (NLS1 and NLS2). IL-5 NLS1$^-$ and NLS3$^-$ mutant β-Gal fusion proteins were found to be excluded from the nucleus, indicating that NLS1 and NLS3 are integral to hIL-5 NLS function. Residues 95-104 of hIL-5 thus appear to constitute the hIL-5 NLS, implying that the IL-5 NLS is not a conventional bipartite NLS.

To investigate the mechanism of uptake of IL-5 into the nucleus, nuclear import of IL-5 and a novel single chain IL-5~HSA fusion protein (hIL-5_{sc}~HSA) was analysed *in vitro* in mechanically perforated rat hepatoma cells. The results of the *in vitro* assays with hIL-5

and mIL-5 indicate that their import does not depend on cytosolic factors, ATP or Ran. Nuclear import was, however, inhibited by wheat germ agglutin (WGA) and excess importin β, suggesting that direct interaction with nuclear pore complexes may play an integral part in the nuclear import process of IL-5. Similar results were obtained for hIL-5_{sc}-HSA, which was derived to exclude the possibility that nuclear import of IL-5, whose size is below the MW cut-off for passive entry through the NPC is via passive diffusion. Nuclear import of hIL-5_{sc}-HSA fusion was independent of cytosolic factors as well as of ATP, and was blocked by WGA. Neither IL-5 nor its fusion protein derivatives were recognized by the conventional nuclear transport machinery. Thus, consistent with the fact that IL-5 does not contain a classical importin α/β recognised bipartite NLS as indicated by the mutational studies, this suggests that IL-5 does not utilize a conventional nuclear transport pathway.

The *in vivo* subcellular fractionation indicated that about 5% of the IL-5 that is internalized reaches the nucleus within 12 hours. This implies that IL-5, and any associated molecules may localize to the nucleus through a unique pathway that allows exit from the endosomes. Previously, piggyback experiments performed with the extracellular domains of the IL-5 receptor α and β subunits suggested that the IL-5 NLS can specifically cotarget the IL-5 receptor into the nucleus (Jans *et al.,* 1997b; Calanni, 1997). Support for the possibility that receptor co-localisation may be important for IL-5 nuclear signalling was obtained in the cell fractionation studies which verified that full length, membrane-integral IL-5 receptor α subunit can be targeted to the nucleus in intact cells, thus verifying results from immunofluorescence studies (Jans *et al.,* 1997a; Calanni, 1997). Nuclear import of the IL-5 receptor a subunit is drastically reduced in the absence of IL-5, but reaches maximum levels within 1 week after re-addition of IL-5, indicating ligand dependence of the IL-5 receptor nuclear targeting. Immunoprecipitation experiments using biotinylated IL-5 showed strong association of IL-5 with its a receptor subunit in the cytosolic fraction, with only a small amount of the receptor subunit associated in the nuclear fraction, suggesting that the ligand/receptor complex may dissociate once it reaches the nuclear compartment of the cell.

Taken together, the results indicate that IL-5 receptor nuclear translocation can occur, in IL-5-dependent fashion, and that the IL-5 receptor subunits may therefore play a role in nuclear signalling. These results support the hypothesis that IL-5's specific signaling role may be to cotransport its receptor subunits to the nucleus through non-conventional nuclear import pathway, where they, either in complex with IL-5 or alone, may affect transcription of genes involved in the activation of eosinophils in allergic disease and parasitic infections. This nuclear signalling may constitute the basis of the specific role of IL-5 in eosinophil differentiation and activation, distinct from IL-3 and GM-CSF, which share common receptor components with IL-5.

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CHAPTER 1 INTRODUCTION

1.1 Interleukin-5 and the IL-3/IL-5/GM-CSF receptor system

1.1.1 Introduction

The ability of multicellular organisms to coordinate cell proliferation, survival, and differentiation is absolutely dependent on the capacity of different cell types to communicate with each other. To enable the large number of cells within an organism to interact without direct cell-cell contact, cells produce a vast variety of signalling molecules. These include polypeptide regulatory molecules such as cytokines, whose primary action is to mediate host defence, and growth factors, whose primary role resides in tissue repair through stimulating cell growth and proliferation. Cytokines and growth factors have the capacity to relay information from a producer cell to a second, responding cell located either in close proximity or at other sites in the body. They do this by binding to specific receptors on the surface of the cell. This in turn triggers a biochemical cascade inside the cell leading to varied biological responses such as motility, adhesion, growth, survival or differentiation (Guthridge, 1998).

More specifically, cytokines are a diverse group of protein molecules, which mediate the effector phases of both innate (non-specific) and adaptive (specific) immunity. Cytokines of the Interleukin 3 (IL-3)/Interleukin 5 (IL-5) and granulocyte/macrophage colony-stimulating factor (GM-CSF) family are important regulators of hematopoiesis through modulation of proliferation, differentiation and survival of various hematopoietic cell lineages and their precursors (Arai, 1990). Whilst IL-3 and GM-CSF act on different blood cells such as granulocytes, macrophages, erythrocytes and early hematopoietic progenitors, the inducible cytokine IL-5 is very specific in its action, and plays a unique role in the regulation of eosinophils. IL-5 is produced by T lymphocytes following activation and is not only essential for the terminal differentiation of eosinophils but is also important for the activation of mature eosinophils (Adachi and Alam, 1998). Eosinophils play a crucial role in the killing of helminth parasites (Meeusen, 2000) and are the most important effector cells for allergic reactions (Adachi, 1998; Sanderson *et al.,* 1988). Understanding the specific signalling pathways of this cytokine is crucial for the development of therapeutic tools for the treatment of conditions like asthma. The identification of the IL-5-specific signalling mechanisms

regulating the function of its target cells is thus of great importance.

1.1.2 Discovery of IL-5

Although the existence of eosinophils as a distinct population of leukocytes was noted more than a century ago, the molecular basis of their functional regulation has only been revealed in the last three decades. Studies examining the role of T cells in the growth and activation of B cells reveal that T-cell supernatants have the capacity to induce B-cell proliferation, leading to the designation of a T-cell replacing factor (TRF) (Schimpl and Wecker, 1972). The B-cell-growth-stimulating activity of T-cell supernatants was attributed to a different factor, called B-cell growth factor, later found to be identical with TRF (Harada *et al.,* 1985). After another factor, responsible for eosinophil differentiation (Sanderson *et al.,* 1985) was co-purified with TRF and the identity of their cDNAs revealed, they were renamed Interleukin-5 (IL-5), functioning as a T-cell-derived cytokine with pleiotropic activities on murine B-cells and human and murine eosinophils (Azuma *et al.,* 1986; Campbell *et al.,* 1988; Kinashi *et al.,* 1986; Sanderson, 1990ab)

1.1.3 Biological function of IL-5

IL-5 has pleiotropic activities on multiple cells as summarized in Table 1.1. Within the IL-3/IL-5/GM-CSF cytokine family, it is the most specific ligand for the eosinophil lineage (Sanderson, 1992). IL-5 plays a central role in determining the number of eosinophils in circulation and in tissues through its ability to promote their production, proliferation, and differentiation and to enhance their survival by suppressing apoptosis. Hematopoietic cytokines are also implicated in the priming of mature eosinophils to a range of stimuli that evoke chemotaxis, degranulation, adhesion, and activation of the nicotinic adenine dinucleotide phosphate (NADPH) oxidase. It is well established that administration of IL-5 to laboratory animals induces blood eosinophilia leading to lung inflammation (Iwama *et al.,* 1992), whilst IL-5-overexpressing transgenic mice show life-long eosinophilia in many organs without overt pathology, indicating that eosinophils require additional factors for activation (Dent *et al.,* 1990).

The primary function of eosinophils is believed to be host defense against infection by

parasitic helminths. This is based on the finding that eosinophil degranulation is directed towards killing helminths *in vitro* resulting in death in the presence of antibodies and complement. They move from the blood to aggregate in the locality of helminths, large numbers of eosinophils often being closely associated with both intact and damaged helminths *in vivo*. They have also been shown to degranulate in the vicinity of helminth surfaces *in vivo* (Butterworth, 1984).

IL-5 seems to play a major role in helminth-induced airway hyperresponsiveness (Hall *et al.,* 1998). Administration of the anti-IL-5 antibody TRFK-5 to mice inoculated with microfilariae of the filarial nematode *Onchocerca lienalis* reduced the ability of the animals to resist reinfection (Folkard *et al.,* 1996). A similar approach has been adopted to show that IL-5 is important in driving eosinophilia and reducing the parasite burden in mice exposed to *Aspergillus fumigatus* (Murali *et al.,* 1993; Kurup *et al.,* 1997), *Toxocara canis* (Buijs *et al.,* 1995), and *Angiostrongylus cantonesis* (Sasaki *et al.,* 1993).

Table 1.1. Targets and biological activities of human and murine IL-5 (from Mahanty and Nutman, 1993)

Target cells	Human	Mouse
	Biological effect	
Eosinophils	Differentiation of precursor cells Prolongation of survival and activation of effector functions of mature cells	
B cells	Role in differentiation and isotype switching is controversial	Differentiation Ig secretion Enhancement of IL-4-directed class switching from IgM to IgG1 and IgE
T cells	Induction of IL-2 receptor expression	Induction of IL-2 receptor expression
Mast cells	Proliferation Histamine release Secretion of leukotriene C4	N.D.[a]

[a] N.D.- not determined

The importance of IL-5 in allergen-induced tissue eosinophilia in laboratory animals has been examined extensively. The exposure of sensitized mice, rats, and guinea pigs to allergen generally results in the appearance of IL-5 and eosinophils in the Bronchoalveolar Lavage Fluid (BAL) fluid. Pulmonary eosinophilia depends on

circulating rather then locally produced IL-5 (Wang *et al.,* 1998) and is associated with an increase in airways reactivity to a variety of stimuli, including acetylcholine (ACh), arecholine, histamine, and 5-hydroxy-tryptamine (Chand *et al.,* 1992; Gulbenkian *et al.,* 1992; Nagai *et al.,* 1993, 1996; Bruijnzeel *et al.,* 1993). Similar effects are seen in the pleural cavity of antigen-challenged sensitized mice (Bozza *et al.,* 1994). Neutralizing IL-5 with antibodies inhibits eosinophil infiltration but has a variable effect on airways responsiveness (Gulbenkian *et al.,* 1992; Chand *et al.,* 1992; van Oosterhout *et al.,* 1993; Nagai *et al.,* 1993, 1996a, 1996b). It thus seems that IL-5-secreting CD4+ Th_2-type cells may play a pivotal role in generating blood and airways eosinophilia in mice, and in the subsequent development of bronchial hyperreactivity and lung damage that occurs in response to aeroallergens (Hogan *et al.,* 1998). Therefore, Il-5 can be seen as a major link between activated T cells and eosinophils in both parasitic as well as in chronic allergic disease (Kotsimbos, 1997) (Fig. 1.1).

IL-5 also appears to play a role in murine B-cell development, being able to induce DNA synthesis in mouse chronic leukemic B cells as well as in dextran-sulfate-stimulated splenic B cells (Hitoshi *et al.,* 1990; Table 1.1). Whilst the B-1 cell population is markedly increased in IL-5 transgenic mice (Tominaga *et al.,* 1991), the number of B-1 cells and peripheral eosinophils in IL-5- and IL-5Rα-deficient mice is reduced (Yoshida *et al.,* 1996; Kopf *et al.,* 1996). Thus, there is evidence for both an IL-5-sensitive developmental pathway for B-1 cells, and a role for IL-5 in the terminal differentiation of mature B-1 cells and activated B-2 cells in the murine system (Takatsu *et al.,* 1994; Table 1.1). Additionally, a synergistic role for IL-5 in promoting C_H gene recombination together with IL-4 has been demonstrated in activated mouse B cells (Mandler *et al.,* 1993). IL-5 is thus a potent cytokine able to induce terminal differentiation of activated mouse B cells, as well as being a priming factor facilitating responsiveness to other cytokines.

In contrast to its role in mouse B-cell development, IL-5's role in human B-cell growth and differentiation remains controversial (see Table 1.1; Huston *et al.,* 1996; Clutterbuck *et al.,* 1987). No effects on proliferation and Ig production were found when IL-5 was tested for functional activity on purified human B cells from peripheral blood (Clutterbuck *et al.,* 1987). Conversely, another study has reported the potential of human B cells to respond to IL-5 (Huston *et al.,* 1996). Detection of mRNA for IL-5Rα

in resting B cells upon the stimulation of B cells with the bacteria B-cell mitogen produced by *Moraxella catarrhalis* and Ig synthesis of B cells stimulated with mitogen and IL-5 have provided direct evidence that human B cells are also able to respond to IL-5 (Huston *et al.*, 1996).

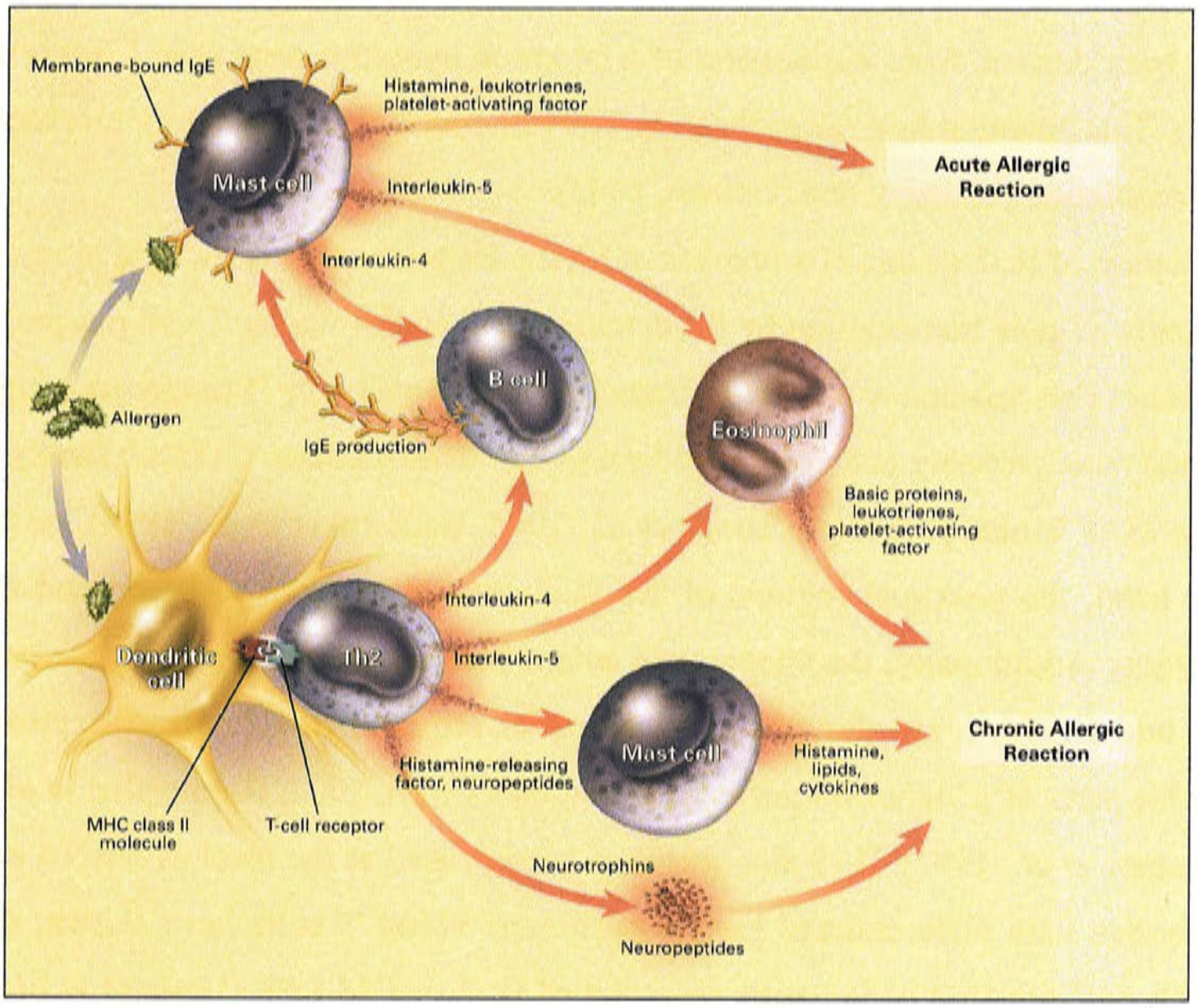


Fig. 1.1. Involvement of IL-5 in allergic reactions.
While **acute** allergic reactions are due to the antigen-induced release of histamine and lipid mediators from mast cells, **chronic** allergic reactions involve the production of IL-5 by activated Th2 cells, causing the recruitment and activation of eosinophils, with subsequent liberation of eosinophil products like basic proteins, leukotrienes and platelet-activating factor. MHC denotes major histocompatibility complex (from Kay, 2001).

1.1.4 IL-5 gene structure and regulation of gene expression

The IL-5 gene is mainly induced in activated T lymphocytes that belong to the Th_2 subset of T cells. The IL-5 gene is part of a cluster of T-helper Th2-type cytokine genes located on chromosome 5 in human (5q23-31) and chromosome 11 in mice. It is found in the vicinity of the genes for IL-3, IL-4 and GM-CSF. The human IL-5 gene has a

length of approximately 4 kb and contains 4 exons and 3 introns, comparable to the mIL-5 gene. The 3 introns of the human IL-5 gene are 208, 950 and 105 bp in length, those of the mouse IL-5 gene are 829, 1875 and 79 bp long. A potential TATA box is located 29 bp (human) and 30 bp (mouse) upstream from the start of transcription (Sanderson *et al.,* 1988). The chromosomal clustering of the IL-5, IL-4 and GM-CSF genes and their inducible expression in T lymphocytes suggests that these genes may have been derived from duplication of a common ancestral gene (van Leeuwen *et al.,* 1989). This potential link raises the possibility of coordinated regulatory mechanisms in their regulation (Mahanty and Nutman, 1993).

Regulation of IL-5 occurs at a number of levels. Its production is thought to result from induction of gene transcription by the cytokine IL-2 and/or via the T cell receptor (TCR) complex (Van Straaten *et al.,* 1994; Valentine and Sewell, 1997) triggering a signalling cascade most probably involving members of the Protein kinase C (PKC) family as well as the MAP kinase pathway (Csonga *et al.,* 1998, Baumruker *et al.,* 1999). At the IL-5 gene level, the proximal regions of the IL-5 promoters seem to be critical for IL-5 induction. Additionally, the cooperative action between the transcription factors AP-1, Ets1 and GATA-3 are thought to be essential requirements for IL-5 expression as possible parts of a higher transcriptional complex (Wan, 2002; Blumenthal *et al.,* 1999, Yamagata *et al.,* 1997). IL-5 also seems to be regulated at the level of mRNA stability. As studies with differentiated human peripheral blood T cells have shown, the IL-5 mRNA half-life ($\geq$ 2 h) is longer than that of IL-3 or GM-CSF (Umland *et al.,* 1998). Only IL-5 transcription initiation shows an absolute dependency on *de novo* protein synthesis (Naora *et al.,* 1994a; Umland *et al.,* 1999).

1.1.5 Biochemical characteristics of the IL-5 protein

The IL-5 protein is a homodimer formed by two identical polypeptide chains joined together by intermolecular disulfide bonds (Minamitake *et al.,* 1990). The crystal structure of hIL-5 was first resolved for a bacterially expressed form (Milburn *et al.,* 1993), followed by the structure of baculovirus-expressed recombinant hIL-5 (Tavernier *et al.,* 1995) and Drosophila-expressed rhIL-5 (Johanson *et al.,* 1995). The structural analysis revealed a novel two-domain structure, with each domain consisting of a four-α-helix bundle (Figs. 1.2 and 1.3).

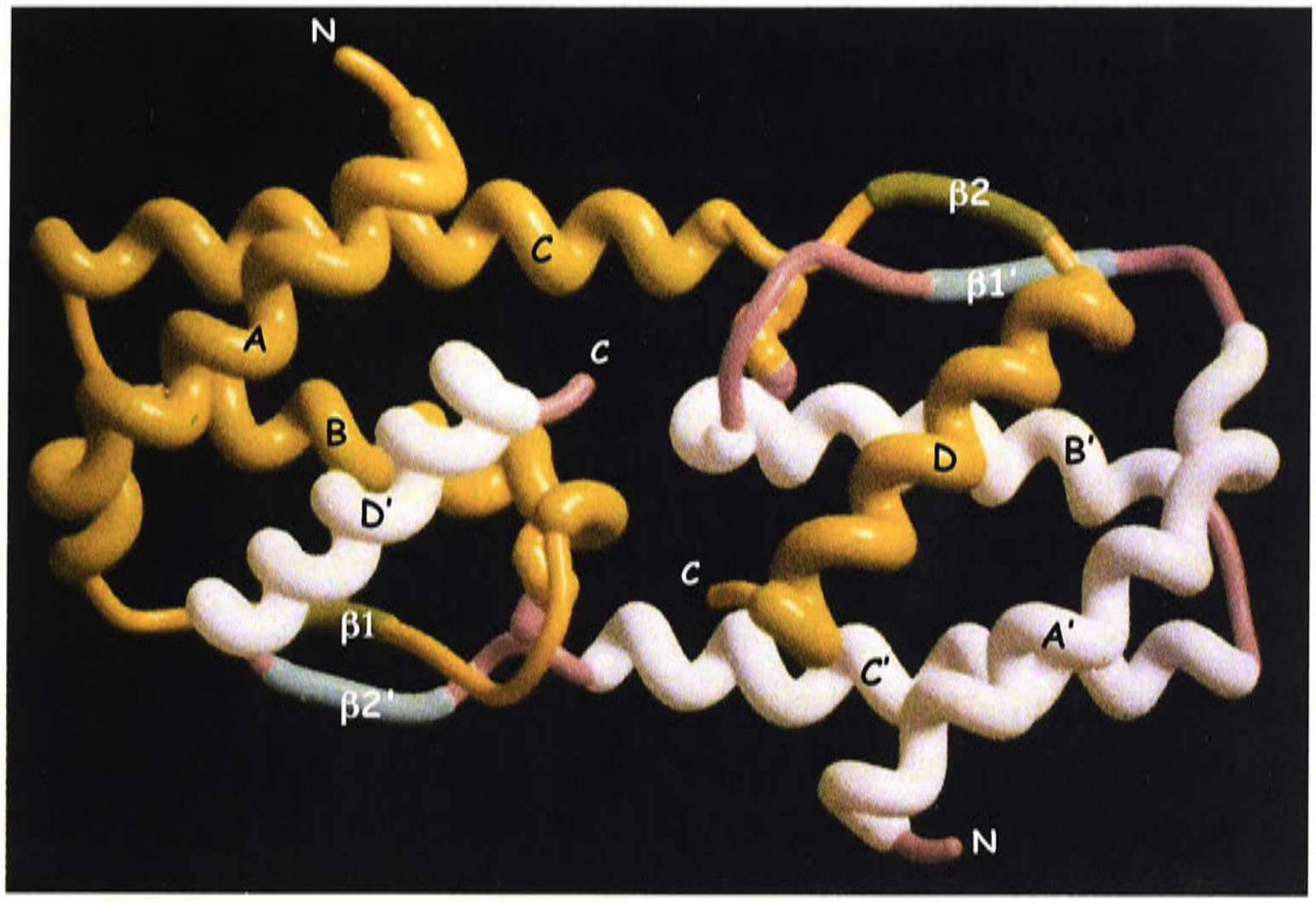


Fig. 1.2. Three-dimensional structure of hIL-5.
Tube drawing of IL-5 showing the dimer configuration. Chain 1 is shown in yellow, chain 2 in white. Light blue and green regions represent the β-sheets of the respective chain. The viewing direction is along the dimer 2-fold axis (from Milburn et al., 1993).

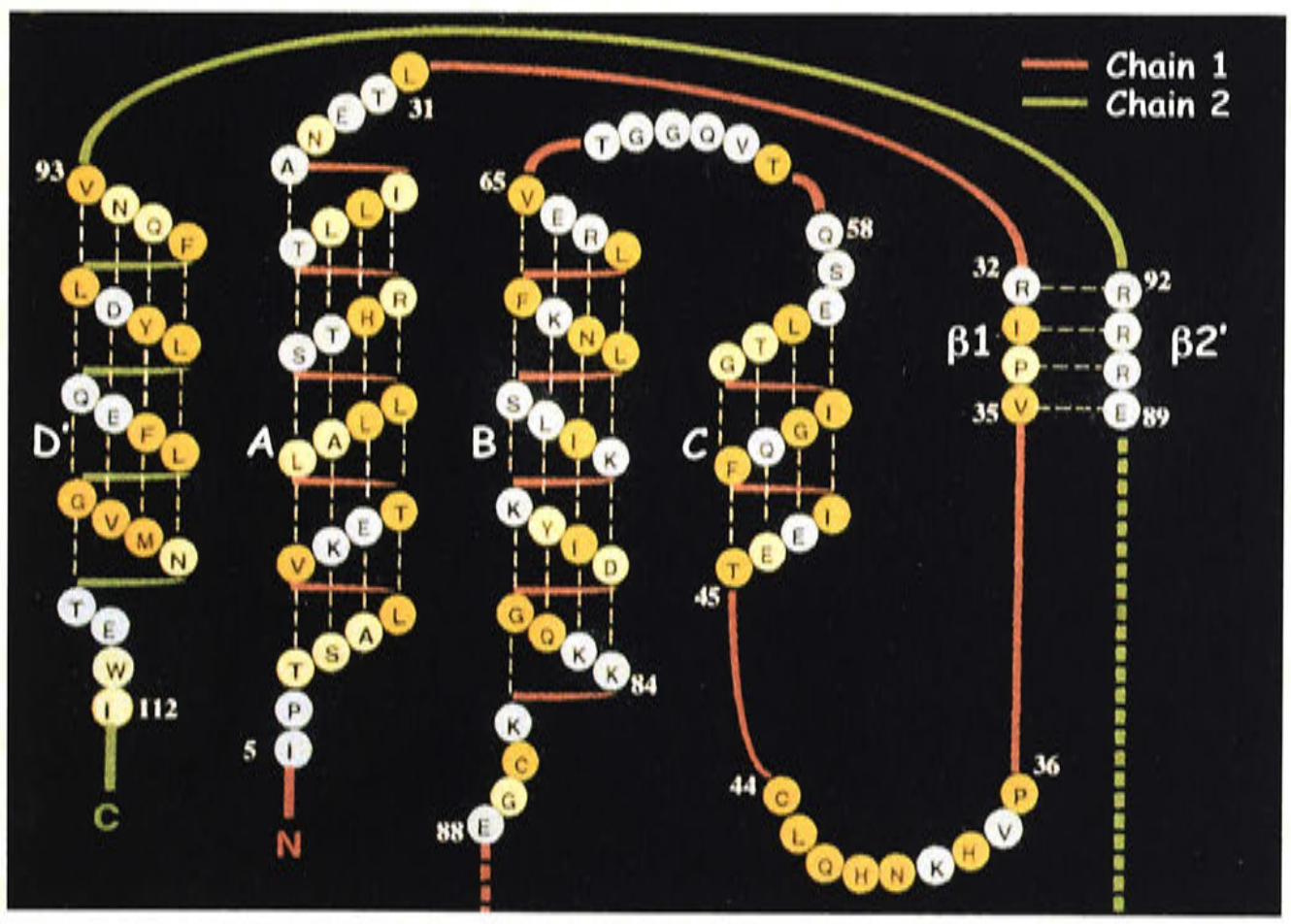


Fig. 1.3. Schematic representation of one human IL-5 domain.
Amino acids are indicated the single-letter code. Each half of the intertwined dimer consists of one monomer chain (red; helices **A**, **B**, **C**) and part of the second monomer chain (green; helix **D'**). The colour code for the individual amino acids reflects side-chain solvent-accessibility: buried residues in orange; intermediate exposed residues in yellow, exposed residues in blue (from Milburn et al., 1993).

The four-helix bundles in IL-5 are composed of three helices from one subunit, with the fourth being provided by the other subunit. These bundles produce an antiparallel dimeric organization unique to IL-5 (Chaiken and Williams, 1996). The intermolecular disulfide bonds linking the two subunits occur in a head-to-tail fashion where the Cys^{44} residue from each chain forms a disulfide with the Cys^{86} residue from the other (Minamitake *et al.,* 1990; Proudfoot *et al.,* 1991). These cysteine residues appear to be essential for IL-5 to function, since their removal by site-directed mutagenesis abolished biological activity of IL-5 (McKenzie *et al.,* 1991). Native IL-5 was initially isolated from mouse T-cell supernatants and shown to be a glycoprotein (McKenzie *et al.,* 1987).

The protein had an apparent molecular mass of 45 kDa, but the cloning of the cDNA sequence revealed that the protein component is composed of two amino acid chains with a molecular mass of 13 kDa for each polypeptide monomer (Kinashi *et al.,* 1986). So far, the sequence of the IL-5 gene of 14 different species was determined.

In hIL-5, an O-linked glycosylation appears at residue Thr^{3} and a N-linked glycosylation at Asn^{28} (Minamitake *et al.,* 1990). Although the carbohydrate components do not appear to be essential for IL-5 activity, it was suggested that the N-linked sugar chains may contribute to the thermostability of the molecule, whilst the O-linked sugar chains seem to be more effective in suppression of IL-5 activity (Kodama *et al.,* 1993). Nevertheless, *Escherichia coli*-expressed rhIL-5 represents a fully active protein, as does that from baculovirus-infected Sf9 cells (Tavernier *et al.,* 1989).

1.1.6 The IL-5 receptor complex

Eosinophils and basophils are the primary target cells of IL-5 in humans, whereas in mice IL-5 also acts on activated B cells (Murata *et al.,* 1992; Mita *et al.,* 1989a; Sanderson *et al.,* 1988; Hitoshi *et al.,* 1990, Tavernier *et al.,* 1991; Chihara *et al.,* 1990; Ingley and Young, 1991). This selectivity in cell activation is due to the fact that only these cells express the receptor system for IL-5. IL-5 has been reported to interact with eosinophils with an affinity of between 20 and 400 pM (Tagari *et al.,* 1993; Lopez *et al.,* 1991; Migita *et al.,* 1991; Ingley and Young, 1991). Also, a broad spectrum of maximum binding sites per cell (260–1500) has been described for cells including primary myeloid cells, eosinophils and basophils, human peripheral blood eosinophils

and human promyelocytic HL-60 cells (Migita *et al.,* 1991; Lopez *et al.,* 1991; Ingley and Young, 1991). The high affinity IL-5 receptor consists of two different polypeptide chains: a 60 kDa IL-5-specific α subunit (IL-5Rα) and a second subunit, the 130 kDa β chain (IL-5Rβ) (Takaki *et al.,* 1990; Takaki *et al.,* 1991; Takaki *et al.,* 1993; Tavernier *et al.,* 1991; Murata et al. 1992). While the α subunit is essential for IL-5 low affinity binding, the β subunit is necessary for high-affinity binding, although it cannot bind IL-5 itself (Fig. 1.4).

Both mouse and human IL-5Rα can occur in soluble forms (Takaki *et al.,* 1990; Murata *et al.,* 1992; Tavernier *et al.,* 1991). Although the function of soluble IL-5Rα is not clear, these receptor forms may be physiological modulators of their cytokine ligands and may alter their effects in hematopoiesis and the immune response (Yamaguchi *et al.,* 1994). It has been suggested that the binding of soluble receptors by cytokines might protect them from proteolytic inactivation and prolong their half-lives *in vivo* (Koike *et al.,* 1994). Recombinant soluble hIL-5Rα (shIL-5Rα) has been shown to compete with the membrane-bound form present on eosinophils and their progenitors and exert antagonistic properties by capturing of IL-5 in the extracellular space (Tavernier *et al.,* 1990; Kikuchi *et al.,* 1994; Daser *et al.,* 2000). shIL-5Rα binds IL-5 with only 2-3 fold lower affinity than the membrane-bound IL-5Rα, and is able to inhibit IL-5-dependent cell proliferation. In contrast, the affinity of soluble mouse IL-5Rα is 10 times lower than the membrane-bound IL-5Rα. Although it can compete for IL-5 binding, it seems to be unable to inhibit IL-5-dependent cell proliferation (Kikuchi *et al.,* 1994)

Little is known of the factors that control the transcription and expression of the IL-5 receptor system (Yasruel *et al.,* 1997; Zanders *et al.,* 1994). At the gene level, no consensus sequences for known transcription factors have been identified in the promoter region of the IL-5Rα gene, indicative of unique myeloid cell- and, possibly, eosinophil-specific, regulatory elements (Sun *et al.,* 1996). The existence of a second promoter for the human IL-5Rα gene was shown to have an important role in the regulation of eosinophil-specific expression of IL-5Rα (Zhang et al. 1997). A recent study showed that IL-5 itself could switch IL-5R α expression predominantly to the transmembrane form in a minigene reporter system (Tavernier *et al.,* 2000).

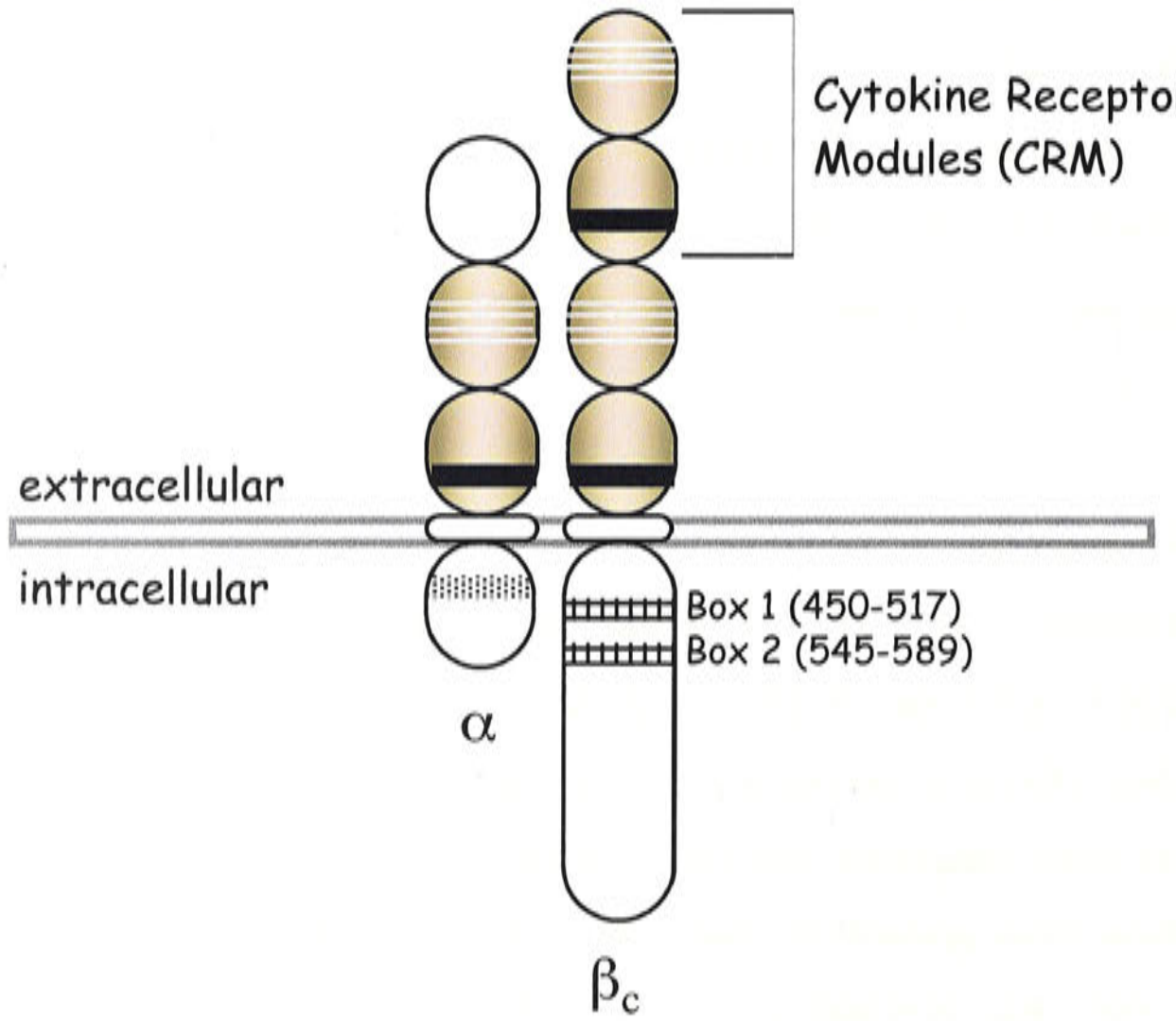


Fig. 1.4. Schematic representation of the IL-5 receptor.
The extracellular portions of IL-5Rα and IL-5Rβ are divided into conserved fibronectin type-III-like domains (shaded) found in the type I cytokine receptor family (Bazan, 1990a). The four conserved cysteines residues are shown as lines, the WSXWS[1] sequence as thick bar. The conserved, proline rich sequence in the cytoplasmic domain of the α chain is shown by a hatched bar.
[1]- amino acids in single-letter code

Structurally, the α and β_c subunits of the Il-5 receptor are members of the Type I cytokine receptor superfamily, which contain conserved extracellular domains termed cytokine receptor modules (CRM) (Fig. 1.4). Each CRM consists of two repeats of a fibronectin type III-like domain. These repeats carry two sets of conserved motifs typical of this family of receptors. The first repeat contains four cysteines with conserved spacing, while the second repeat contains a WSXWS (single letter amino acid code) motif (Bazan, 1990a).

The extracellular portion of IL-5Rα is composed of three fibronectin type-III-like modules (Takaki *et al.*, 1990; Murata *et al.*, 1992). The two membrane proximal modules contain the conserved superfamily structures; four conserved cysteines in the middle domain and the WSXWS motif (Bazan *et al.*, 1990a) in the membrane proximal

domain. The cytoplasmic domain of IL-5Rα has no significant homology with signalling molecules such as kinases, phosphatases, or nucleotide binding proteins, and no Src homology (SH) domains. It contains cytoplasmic regions rich in proline (Pro-Pro-X-Pro) following the transmembrane domain that are well conserved between IL-5Rα and the receptors for IL-3, GM-CSF, prolactin, and growth hormone (Takaki *et al.,* 1990).

The crystal structure of the human β_c subunit has recently been resolved (Carr *et al.,* 2001). The β_c subunit appears to be an intertwined dimer in which all subunits consist of four domains, each with an approximate fibronectin type III topology. The β_c dimer structure seems to be analogous to that of the IL-5 dimer, where helix D and strand 2 appear to have been exchanged as a result of an ancestral domain swapping event that has subsequently undergone evolutionary selection, resulting in a short hinge loop between helix C and D. The latter prevents helix D from packing close to helices A-C, thus precluding the formation of a stable monomeric species (Milburn *et al.,* 1993).

IL-5 has significant functional homology with IL-3 and GM-CSF, all of which utilize the receptor β subunit (β_c), with the ligand specificity preserved by distinct α receptor subunits. After ligand-binding, the β_c subunit, which is crucial for signal transduction (Adachi et al. 1998), forms a high affinity receptor with all three α subunits.

1.1.7 Transmembrane signalling by the IL-3/IL-5/GM-CSF receptor system

The exact nature of the signalling pathways involved in the activation of hematopoietic cells by IL-3/IL-5/GM-CSF is not well established. Nevertheless, these cytokines are known to activate at least three common pathways: the Janus kinase family (JAK)/ Signal transducer and activators of transcription (STAT) pathway, the ras/Mitogen activated protein kinase (MAPK) pathway, and the Phosphatidyl-inositol-3 kinase (PI3-K) pathway (Fig. 1.5).

Although the β_c receptor subunit of IL-3, IL-5 and GM-CSF lacks intrinsic kinase activity, these ligands induce tyrosine phosphorylation of β_c as well as a variety of cytoplasmic proteins including kinases (Welham *et al.,* 1994), adaptor proteins (Okuda *et al.,* 1997), guanine nucleotide exchange factors (Matsuguchi *et al.,* 1995),

phosphatases (Welham *et al.,* 1994), and transcription factors (Mui *et al.,* 1995). This phosphorylation is mediated mainly by the receptor-associated JAK2. Janus family kinases are cytoplasmic tyrosine kinases that are associated with cytokine receptors both in vertebrates and invertebrates (Mathey-Prevot *et al.,* 1998).

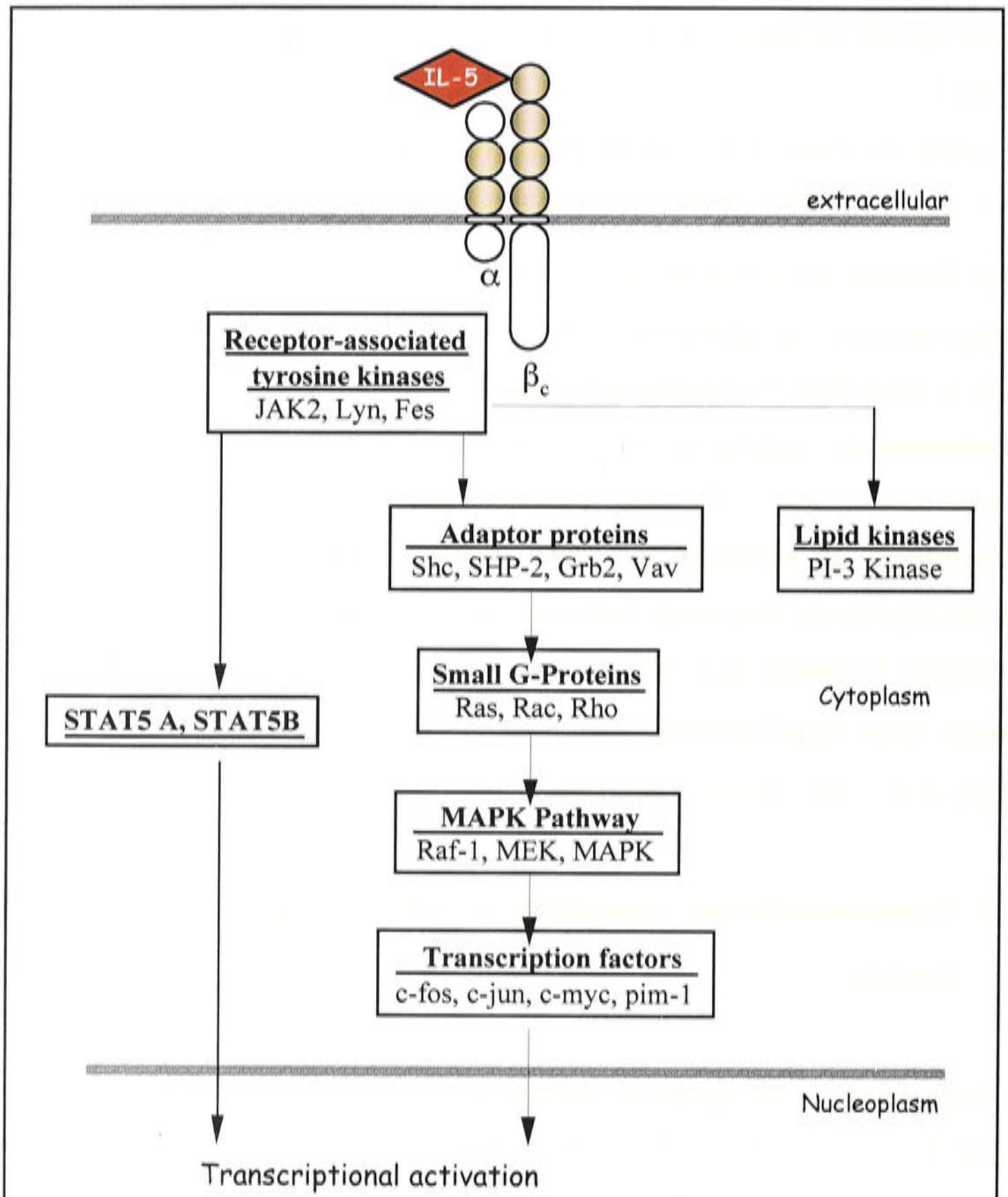


Fig. 1.5. Schematic representation of signal transduction pathways activated through IL-3/IL-5/GM-CSF receptors.
Receptor activation following ligand binding leads to activation of multiple cytoplasmic signalling molecules, thought to leads ultimately to proliferation, differentiation, survival and activation of cellular functions (adapted from Adachi and Alam, 1998).
STAT - Signal transducers and activators of transcription; JAK - Janus family kinase.

The membrane proximal region of the β_c subunit contains a conserved proline-rich motif termed box 1, which serves as a binding site for JAK2 (Quelle *et al.,* 1994). Upon ligand binding and following receptor dimerization, the JAKs are activated by trans-phosphorylation of two receptor-bound JAK molecules and subsequently phosphorylate a number of substrates including the cytokine receptor itself.

Following receptor activation and phosphorylation, phosphotyrosine residues within the cytoplasmic domain of the β_c receptor function as high affinity binding sites for SH-2 domain-containing proteins. These proteins include the STAT5 homologues STAT5A and STAT5B, members of the family of cytoplasmic transcription factors (Ihle, 1996, Darnell, 1997). Phosphorylation of STAT5 by JAK2 then results in STAT5 activation, dimerization and translocation to the nucleus, where it is directly involved in regulating gene transcription (Itoh *et al.,* 1998; Mui *et al.,* 1995). The Extracellular signal-regulated kinases (ERK) 1 and 2 members of the MAPK family have been shown to be activated by IL-3, IL-5 and GM-CSF in primary cellular lineages as well as in cell lines (Pazdrak *et al.,* 1995b; Coffer *et al.,* 1998). Rapid activation of the guanine nucleotide binding protein Ras (p21) induces translocation of Raf-1 kinase to the juxtamembranous compartment, where the latter is activated by tyrosine kinases, protein 14-3-3, and possibly Protein kinase C (PKC) (Stomski *et al.,* 1999). Raf-1 regulates ERK, a member of the MAPK family, through activation of MEK (MAP or ERK kinase). Activation of the raf-1/MAPK pathway eventually results in enhanced transcription of c-Fos and c-Jun (Satoh *et al.,* 1991; Sato *et al.,* 1993) and might contribute to IL-3, IL-5 and GM-CSF induced proliferation.

IL-5 can also activate the serine/threonine protein kinase Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway (de Groot *et al.,* 1997). Downstream targets of this pathway are likely to be transcription factors involved in c-Jun and c-Fos regulation such as ternary complex factor (TCF), and activating transcription factor-2 (ATF2), as well as c-Jun itself (de Groot *et al.,* 1997).

Additionally, IL-5 stimulation results in rapid tyrosine phosphorylation of PI3K (Sato *et al.,* 1994). The β_c receptor does not contain a consensus motif for PI3K binding, and it is likely that other proteins serve as bridging intermediate. Possible candidates are SHP-2 (Welham *et al.,* 1994) and Tec tyrosine kinase (Takahashi *et al.,* 1997). PI3K

transduces signals to a number of serine/threonine kinases such as PKC, protein kinase B (PKB or Akt), JNK and p70 S6 kinase (de Groot *et al.,* 1997).

1.1.8 Functional role of IL-5/IL-3/GM-CSF signalling pathways

The functional role of the signalling pathways used by IL-3/IL-5/GM-CSF for proliferation, survival and differentiation of different target cells have only recently been characterized (Table 1.2).

Table 1.2. Role of IL-5 on eosinophils and the intracellular signals involved [1].

Role of IL-5	Signalling molecules
Eosinophilic progenitors	
Proliferation and Differentiation	Lyn, JAK2
Mature eosinophils	
Inhibition of apoptosis	Lyn, Syk, JAK2, SHP-2, Raf-1
Priming for degranulation, production of leukotriene and superoxide	PI3K, Raf-1
Direct degranulation and superoxide production	N.D.
Stimulation of cytokine synthesis	N.D.
Upregulation of adhesion molecules and induction of adherence	Raf-1
Stimulation of cytotoxicity	N.D.
Expression of MHC class II	N.D.
Chemotaxis	N.D.

N.D.- not determined
[1] (Adachi and Alam, 1998)

Most of the work connecting the IL-3/IL-5/GM-CSF signalling pathways to physiological function has been performed by analysis of the β_c receptor subunit in factor-dependent cell lines. Deletion and point mutagenesis of the cytoplasmic domain of β_c have demonstrated that JAK2, known to bind to the box 1 region, is both necessary and sufficient to induce proliferation in mouse Ba/F3 pro-B and CTLL2 cytotoxic T cells (Itoh *et al.,* 1996; Watanabe *et al.,* 1996).

The box 2 region in β_c enhances proliferative responses, but is not absolutely required. So far, no single tyrosine residue at β_c has been found to be crucial for mediating any of the known biological activities of IL-3/IL-5/GM-CSF, which implies a high degree of

redundancy. When all 8 tyrosine residues in β_c were replaced, proliferation was not impaired, suggesting that additional pathways might be involved in proliferative signalling. IL-3/IL-5/GM-CSF also induce signals leading to prolonged survival and inhibition of apoptosis of their respective receptor-bearing cells (Guthridge *et al.,* 1998). Although JAK2 activation is required for all known signalling pathways originating from β_c, its importance in anti-apoptotic signalling remains to be proven. Tyrosine-phosphorylation does not seem to be essential, as indicated by mutational analysis (Okuda *et al.,* 1997; Itoh *et al.,* 1998).

The recent discovery of a novel PI3-kinase pathway has provided new insights into how cytokines can promote survival by the prevention of apoptosis (Franke *et al.,* 1997). In the case of IL-3 stimulation of Ba/F3 cells at least, the activation of the PI3-kinase ultimately seems to lead to binding of the adapter protein 14-3-3 to BAD (**B**cl-2/Bcl-X L -**A**ntagonist, causing cell **D**eath). This possibly prevents BAD from exerting its pro-apoptotic activity, leading to IL-3-mediated survival (Zha *et al.,* 1996; del Peso *et al.,* 1997).

In vitro studies with human blood eosinophils have suggested that IL-5 may act by altering expression of the anti-apoptotic regulator Bcl-2 and its homologues (Dewson *et al.,* 1999; Dibbert *et al.,* 1998), possibly through cross-talk with the PI3-K/Akt and the STAT5 pathways (Santos *et al.,* 2001), but this remains controversial. Alternatively, IL-5 may act by regulating the activation of caspase cell death cascades rather then altering the balance of anti-apoptotic and pro-apoptotic Bcl-2 homologues (Zangrilli *et al.,* 2000). This model was supported by a recent report demonstrating that caspase-8 and 3 are the main targets of IL-5 prolonging survival as mediators of Fas-induced eosinophil apoptosis (Letuve *et al.,* 2001). Recently, NF-κB was shown to be involved in TNF-alpha-enhanced eosinophil survival through the regulation of GM-CSF production by eosinophils, with MAPK inhibitors having no effect on this pathway (Temkin and Levi-Schaffer, 2001). Mutational analysis of β_c indicates that, at least in the case of GM-CSF, an intracellular domain including the box 3 region is essential for GM-CSF-mediated differentiation of M1 cells (Smith *et al.,* 1997). It has been demonstrated for eosinophils that the tyrosine kinases Lyn and Jak2 play an essential role in IL-5Rα signalling, leading to eosinophil differentiation (Stafford *et al.,* 2002). Lyn was directly associated with IL-5Rα, and the effect of Lyn appeared to be relatively specific for the eosinophilic lineage. To date, however, the critical role of IL-3/IL-5/GM-CSF in

differentiation cannot be explained on the basis of the current knowledge of signalling pathways elucidated for these cytokines.

Little is known about the effects of above described signalling pathways on myeloid cell effector function such as cell migration, priming, degranulation or induction of adherence. PI3 kinase, but not Ras-MAPK, appears to have a role in superoxide production in IL-5-primed cells (Coffer *et al.,* 1998). Lyn and JAK2 seem not to be important for degranulation, whereas Raf-1 kinase has been demonstrated to play a central role in regulating not only for eosinophil survival, but also for expression of β2 integrins as well as for degranulation (Pazdrak *et al.,* 1998).

1.1.9 Determinants of signalling specificity in the IL-3/IL-5/GM-CSF ligand-receptor system

The sharing of a common receptor subunit (β_c) has profound implications for the defining of key events that distinguish the signalling pathways between IL-3/IL-5/GM-CSF. Tan and Kim (1999) have summarized several proposed models for how particular molecular mechanisms can achieve signalling specificity in the RTK/Ras/MAP kinase signalling pathway, which can reasonably apply to other pathways as well, including those activated by the cytokine type I receptor family.

Model 1: Differential signalling kinetics. Differences in the level of activation of a common signalling pathway might generate different cellular outcomes, either by stimulating the same receptor to different degrees, or by activating other receptors (or subunits of the same receptor system) that feed into identical intracellular signalling pathways.

Model 2: Integration of multiple signalling pathways. Signals from separate and distinct signalling pathways might be integrated by common proteins, affecting the decision of a tissue or cell to execute a particular cell fate.

Model 3. Cell-type or cell-stage-specific downstream effectors. Cells of different types or cells at different developmental stages might express distinct proteins that respond to the same upstream pathway to generate different cellular outcomes.

The basic principle of how specificity is reached differs between the models. In Model 1 and 2 signalling specificity is controlled by the levels or types of "instructive" cellular signals acting on cells or tissues in a "tissue-extrinsic" fashion, while Model 3 shows how the expression of cell or tissue-specific traits can be determined by inherent differences in the responding cells or tissues. Here, cellular signals function in a "permissive" manner by triggering distinct responses that are pre-programmed in cells of different origin or at different developmental stages, and signalling specificity is controlled in a "tissue-intrinsic" fashion (Tan and Kim, 1999). It is possible that similar mechanisms are responsible to ensure signalling specificity in the IL-3/IL-5/GM-CSF system.

Different cells of the hematopoietic system may express or lack one or several of the ligand-specific α receptor subunits at different times during maturation and activation. Specifically, the IL-3 receptor is known to be expressed on early hematopoietic progenitor cells and certain committed myeloid progenitors, as well as on eosinophils and basophiles. The GM-CSF receptor is expressed on most types of myeloid progenitors, mature monocytes, neutrophiles, eosinophiles, basophiles and dendritic cells. In contrast, the IL-5 receptor seems to be expressed exclusively on eosinophils, basophils and murine B cells (Nicola, 1994).

Model 1 suggests that subtle differences in the level of receptor expression could result in marked differences in activation of down-stream signalling components. As Rossi et al. (1996) have demonstrated, the lineage commitment of a hematopoietic precursor cell line can be controlled by the level of PKC activity. While the absence of PKC activity maintained cells in an undifferentiated state, low PKC activity induced differentiation towards a myelomonocytic phenotype; high PKC activity, in contrast, favored eosinophilic differentiation. In addition, the level of ligand concentration at a particular time appears to be able to determine the lineage to which a particular progenitor cell differentiates. It has been reported for example, that high IL-3 concentrations promote the self-renewal of a murine multipotential hematopoietic cell line, whereas low concentrations in combination with GM-CSF result in differentiation towards granulocytes, macrophages or erythroid cells (Heyworth *et al.,* 1990).

With ligands like IL-3/IL-5/GM-CSF sharing a receptor subunit thought to be the main signalling entity, the specific α receptor subunit may not be important solely for ligand recognition and high affinity binding, but might also be involved in ligand-specific

signalling and recruitment of specific intracellular adaptor molecules and signalling pathways. Experimental evidence is now accumulating that the α receptor subunits of the IL-3/IL-5/GM-CSF system may possibly play a role in signalling, despite only bearing a 50 amino acids long intracellular domain. Mire-Sluis et al. (1995) claim to have demonstrated that IL-5-induced proliferation in the cell line TF-1 is inhibited by the phosphatase inhibitor sodium orthovanadate with no effect on IL-3/GM-CSF induced proliferation. Similarly, although IL-3/GM-CSF induced proliferation is inhibited by the tyrosine kinase inhibitor genistein, IL-5-dependent proliferation seems to be unaffected.

Previously, a GM-CSF receptor α subunit-associated protein (GRAP) was isolated using a yeast-2-hybrid system and co-immunoprecipitation studies in mammalian cells (Tu *et al.,* 2000). GRAP appears to play an important role in cellular metabolism, and it was shown that GRAP does not interact with the IL-3Rα subunit, indicating specificity in the interaction of GRAP with the GM-CSFRα, though binding of IL-5Rα was not tested. Similarly, JAK2 has been shown to interact with the IL-5Rα Box 1 region, a proline-rich domain highly conserved between the IL-3/IL-5/GM-CSF receptor α subunits. Interaction with the IL-3 or GM-CSF receptor α subunit, however, has not been shown (Ogata *et al.,* 1998). Most recently, a direct association of an adapter protein called syntenin with the IL-5Rα subunit was reported (Geijsen *et al.,* 2001). Sytenin was found to be associated exclusively with the cytoplasmic tail of IL-5Rα, and did not bind to the IL-3 or GM-CSF subunits. Syntenin directly associated with the transcription factor SOX4, whose IL-5-mediated activation followed the binding of Syntenin to the IL-5Rα.

Despite the above studies, the signalling pathways currently defined for IL-5 do not account for its specific effect on the activation and differentiation of eosinophils, since all of these pathways are also activated by IL-3 and GM-CSF. It is therefore possible that the specific actions of IL-5 may either be due to mechanisms ensuring signal specificity as described above, or it may be due to the existence of other novel signalling pathways specific to IL-5.

One feature of the IL-5 molecule consistent with the idea that additional signalling pathways may be responsible for the specific functions of IL-5 is the fact that both human and mouse IL-5 contain a putative nuclear targeting signal (NTS), resembling a

bipartite nuclear localization signal (NLS) (Jans, 1994; Jans *et al.*, 1997a). This suggests that IL-5 could be directly involved in signalling in an "instructive" manner. NLSs have been identified for a variety of polypeptide ligands, including cytokines and growth factors, implying that these ligands can be targeted to the nucleus where they may have an important role in signalling. The next sections will summarize the current state of knowledge for nuclear transport in eukaryotic cells and review the data currently available supporting the concept of nuclear localization of polypeptide hormones, growth factors and their receptor systems as an additional mechanisms to achieve signalling specificity.

1.2 General mechanisms of nuclear transport

1.2.1 Nuclear-cytoplasmic processes in the eukaryotic cell

The fact that eukaryotic cells possess a nucleus carries important implications for the flow of information between the genome of a cell and its cellular functions, meaning that its genetic information, the DNA, is separated from the site of protein synthesis, the cytoplasm. Separation is effected by a double membrane structure, the nuclear envelope (NE), and as a result, gene transcription and translation take place in different subcellular compartments, meaning that specific transport events between the two are necessary for the cell to function. mRNA must make its way from the nucleus to the cytoplasm in order to be translated into protein, whilst proteins needed in the nucleus, including the enzymes required for DNA replication, gene regulation, and transcription, have to be transported from their site of synthesis in the cytoplasm to the nucleus. All transport goes through the NE-localized nuclear pore complexes (NPC), which have a molecular sieve function (see 1.2.2). Molecules smaller than 40–45 kDa can diffuse freely between cytoplasm and nucleus, while larger molecules require specific targeting signals (NLS's – see Section 1.2.3) in order to enter the nucleus. This provides the cell with a means to regulate the process of protein transport to the nucleus and make it highly specific. In terms of signal transduction leading to phenotypic changes such as differentiation, proliferation, and transformation, the cytosolic signals transduced at the level of the membrane through receptor activation upon hormone, growth factor, or cytokine binding need to be converted into nuclear signals. Almost without exception, this involves protein translocation events from the cytoplasm to the nucleus (Jans and Hassan, 1998).

1.2.2 The nuclear pore complex (NPC)

Spatial connection between the nucleus and cytoplasm is provided by a large protein assembly termed the NPC, embedded in the NE (Feldherr *et al.*, 1984; Rout *et al.*, 2000). The number of NPCs per cell appears to depend on the demand for nuclear transport, and accordingly varies greatly with cell size and synthetic and proliferative

activity. There are approximately 200 NPCs in a yeast cell (Rout and Blobel 1993), 3000–5000 in a proliferating human cell, and 5 x 10^7 in a mature *Xenopus* oocyte (Cordes et al 1995).

The NPC is a large structure, varying in size from 60 MDa in *S. cerevisiae* to 125 MDa in vertebrates, which contains multiple copies of 30 to 50 different proteins called nucleoporins (Nups) (Stoffler *et al.,* 1999; Rout *et al.,* 2000) in varying stoichiometries.

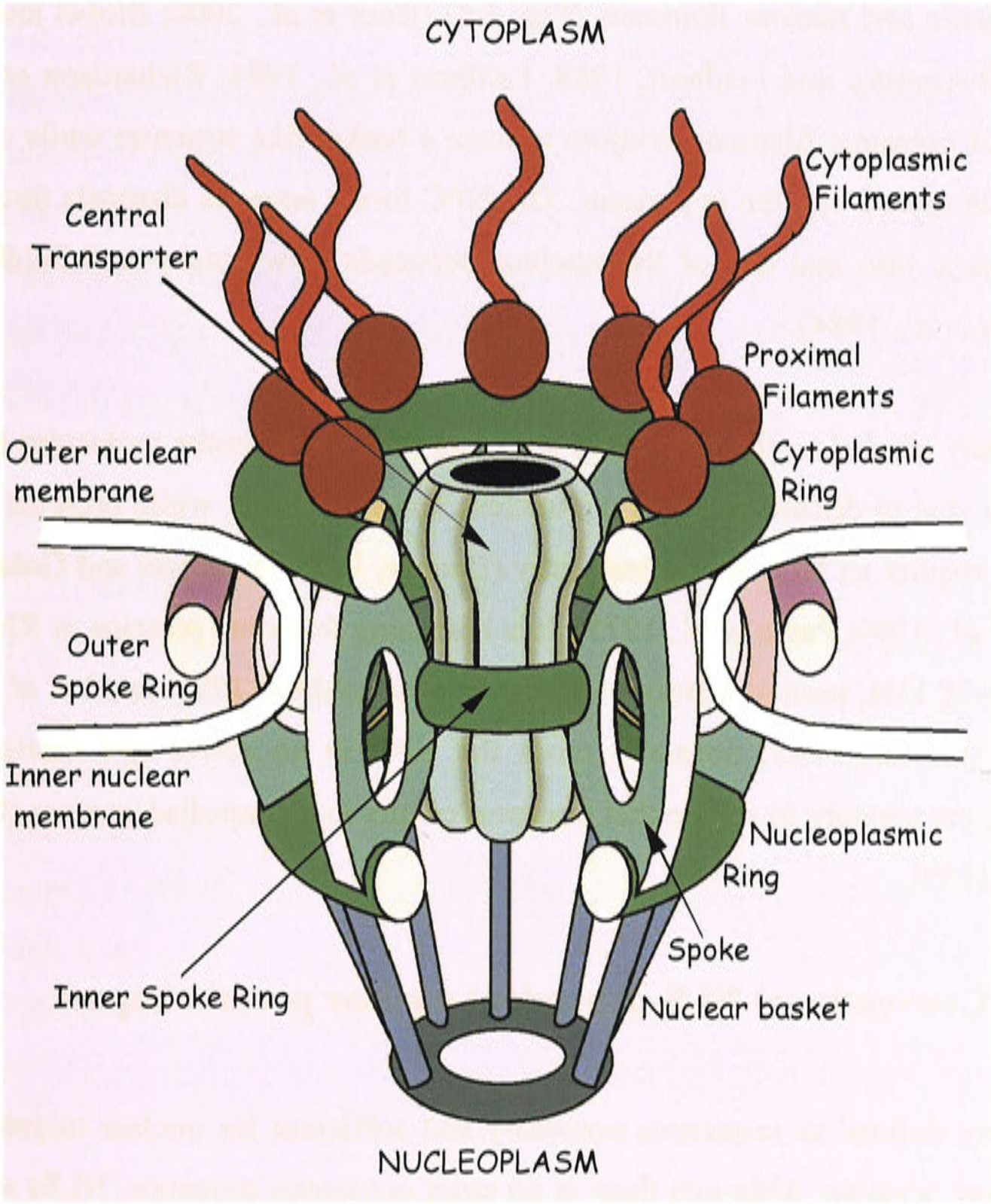


Fig. 1.6. Model of the nuclear pore complex.
The NPC consists of about 30 different proteins, forming a basic subunit that is repeated 16 times. Additional, filamentous structures are on both sides of the pore. On the nuclear side, these link together to form the nuclear basket (Blobel and Wozniak, 2001).

Although the overall sequence homology between yeast and vertebrate Nups is usually not greater than 30% identity (Ryan and Wente, 2000), NPCs are highly conserved in both structure and function across eukaryotes implying their central role in the transport process (Davis, 1995; Fabre and Hurt, 1997). Nups are classically defined as being stably associated with the NPC, although mobile Nups have more recently been identified (Nakielny *et al.,* 1999; Zolotukhin and Felber, 1999).

The basic structural framework of the NPC consists of an 8-fold rotational symmetry whereby 8 interconnected spokes surround a central gated channel with attached cytoplasmic and nuclear filaments (Fig. 1.6) (Rout *et al.,* 2000; Blobel and Wozniak, 2001; Dworetzky and Feldherr, 1988; Feldherr *et al.,* 1984; Richardson *et al.,* 1988). The nucleoplasmic filaments conjoin to form a basket-like structure while cytoplasmic filaments spread into the cytoplasm. The NPC forms aqueous channels through which all passage into and out of the nucleus proceeds (Dworetzky and Feldherr, 1988; Feldherr *et al.,* 1984).

As already alluded to, the NPC has a pore-like function whereby molecules less than 40 kDa are able to diffuse between the nucleus and cytoplasm, while proteins larger than 40 kDa require an NLS for nuclear entry (Bonner, 1975; Breeuwer and Goldfarb, 1990; Jakel *et al.,* 1999; Paine *et al.,* 1975). It is intriguing that even proteins or RNAs smaller than 20–30 kDa, such as histones (Breeuwer and Goldfarb 1990, Jakel *et al.,* 1999) and tRNAs (Zasloff 1982) normally cross the NPC in an active and carrier-mediated fashion, presumably to ensure that transport occurs in a controlled manner (Görlich and Kutay, 1999).

1.2.3 Conventional NLS-dependent nuclear protein import

NLSs are defined as sequences necessary and sufficient for nuclear targeting of their respective proteins. Although there is no strict consensus sequence, NLSs are typically small regions of basic amino acids (Dingwall and Laskey, 1991) arranged either in a single cluster (monopartite) as typified by that of the well-characterized Simian Virus (SV40) large tumor antigen (T-ag) (Kalderon *et al.,* 1984a/b; Lanford and Butel, 1984) (Table 1.3 A), or situated in two clusters separated by a 10-12 amino acid spacer (bipartite) resembling the NLS of the *Xenopus laevis* phosphoprotein nucleoplasmin

(Table 1.3 B) (Chelsky *et al.,* 1989; Robbins *et al.,* 1991).

In contrast to other transport signals such as those for mitochondrial or ER localization, NLSs are not cleaved after transport, as they are required to function several times through a number of cell divisions, following the open mitosis of eukaryotic cells. In addition, as NLSs are not removed (Agutter and Prochnow, 1994; Nigg, 1997; Stochaj and Silver, 1992) nor confined to N- or C-terminal regions of the protein, the only restriction regarding the position of an NLS within the protein is that they are accessible.

Two experimental approaches for identification of functional NLS regions are:

1) Mutagenesis of residues of an NLS results in cytoplasmic localization of the NLS-containing protein, indicating that the NLS is necessary for nuclear import.
2) Coupling of an NLS to a normally cytoplasmic protein either as a fusion protein or as a cross-linked peptide, targets the protein to the nucleus, indicating that the NLS is sufficient for nuclear import.
3)

Two experimental setups are commonly used to investigate the transport properties of NLS-bearing proteins; one using transfection, and the other utilizing labelled proteins. The transfection approach does not provide information regarding the rates of nuclear protein import, and the high levels of protein that are generally expressed may not represent physiological conditions (Jans *et al.,* 1995b). A different approach uses fluorescently labelled proteins, which are microinjected into the cytoplasm of single living mammalian cells prior analysis of nuclear import by confocal laser scanning microscopy (CLSM).

CLSM has been used quantitatively in combination with the microinjection of fluorescently labelled proteins to resolve the nuclear protein import process temporally. The establishment of *in vitro* or reconstituted nuclear import assays has greatly enhanced our knowledge of nuclear transport at the molecular level. These assays have been based on permeabilizing the cytoplasmic membrane of mammalian cells (Adam *et al.,* 1990, 1992) utilizing digitonin, a detergent that selectively permeabilizes the cholesterol-rich plasma membrane without dramatically affecting the nuclear membrane.

Table 1.3 A. Selected examples of NLS's resembling the monopartite NLS of T-ag.

Protein	NLS[1]	References
Viral Proteins		
T-ag	PKKKRKV [132]	Kalderon et al. (1984a, b); Lanford and Butel (1984); Yoneda et al. (1987)
Polyoma T-ag	VSRKRPRP [196]	Chelsky et al. (1989); Richardson et al. (1986)
SV40 VP2/3	PNKKKRK [323]	Clever and Kasamatsu (1991)
Transcription factors		
Dorsal	RRKRQR [340]	Govind et al. (1996)
v-jun	KSRKRKL [253]	Chida and Vogt (1992)
NF-AT	CNKRKYSLN [271]	Beals *et al.*, (1997)
NF-kB p50	QRKRQK [372]	Blank et al. (1991); Henkel et al. (1992)
Nuclear Proteins		
Lamin L I	VRTTKGKRKRIDV [420]	Chelsky et al. (1989)
Lamin B2	RSSRGKRRRIE [419]	Hennekes et al. (1993)
yeast Histone 2B	GKKRSKAK [36]	Moreland et al. (1987)
Other Proteins		
Basonuclin	PKKKSRKSS [541]	Iuchi and Green (1997)
Cofilin	PEEVKKRKKAV [36]	Abe et al. (1993)

[1] NLSs are shown in the single letter amino acid code with numbers referring to the residue number in the protein sequence. NF-AT, Nuclear factor of activated T-cells; SV 40 T-ag, Simian virus 40 large tumor antigen; SV 40, VP2/3- simian virus 40 (SV40) structural protein VP2/3;

Table 1.3 B. Selected examples of NLSs resembling the bipartite NLS of nucleoplasmin.

Protein	NLS[1]	References
Nuclear Proteins		
Nucleoplasmin	KR -9 aa spacer- KKKKL [171]	Chelsky et al. (1989); Lanford et al. (1990); Robbins et al. (1991)
Xenopus NO38	KR -11 aa spacer- KKTR [153]	Peculis and Gall (1992); Robbins et al. (1991)
RB	KR -11 aa spacer- KKLR [869]	Efthymiadis et al. (1997)
hpoly(ADP-ribose) polymerase	KRK -10 aa spacer- KKKSKK [226]	Schreiber et al. (1992)
N1N2	RKKRK -12 aa spacer- KKSK [551]	Kleinschmidt and Seiter (1988)
Transcription factors		
human Estrogen receptor	RK -11 aa spacer- RKDR [495]	Picard et al. (1990); Ylikomi et al. (1992)
human ARNT	RAIKRR -13 aa spacer- KFLR [61]	Eguchi et al. (1997)
SWI5	KK -10 aa spacer- RKRGRPRK [655]	Jans et al. (1995a); Moll et al. (1991)

[1] NLSs are shown in the single letter amino acid code with numbers referring to the residue number in the protein sequence.RB, retinoblastoma; ARNT, aryl hydrocarbon receptor nuclear translocator;

The same effect can be achieved by mechanical perforation of the plasma membrane (Jans *et al.*, 1991) to allow the tracking of fluorescent import substrates and the monitoring of their nuclear uptake by CLSM. Since the cells are depleted of their cytosolic components in this approach, the addition of exogenous cytosolic fractions or recombinant proteins provides a means of identifying essential, soluble transport factors.

NLS-dependent nuclear protein import proceeds by an ordered process dependent on numerous cytosolic factors including proteins of the NLS-recognizing importin superfamily and the monomeric GTPase Ran. Conventionally, the process can be divided into five main steps (Catimel *et al.*, 2001; Görlich and Mattaj, 1996; Nigg, 1997; see Fig. 1.7):

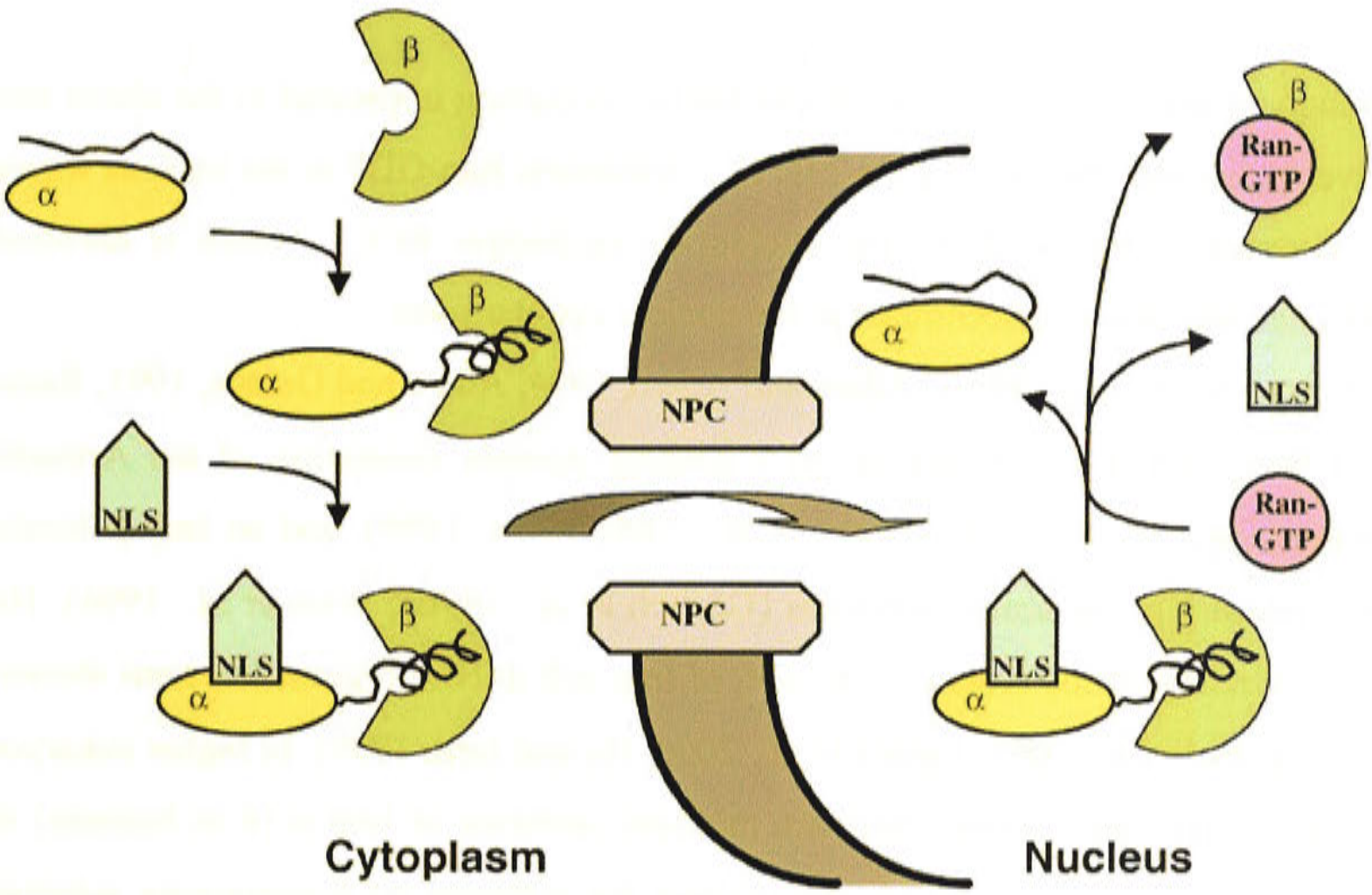


Fig. 1.7. Conventional NLS-mediated nuclear protein import. After release of the NLS binding site of Impα and subsequent Impβ-binding to the IBB domain of Impα, a protein containing an NLS is recognised by the Impα/β complex through Impα. Impβ mediates binding to the NPC and the complex is translocated into the nucleus. Once in the nucleus, Ran-GTP binding to Impβ causes dissociation of the transport complex and the cargo is released into the nucleoplasm (adapted from Catimel et al., 2001).

1) Binding of importin β (Imp β) to an Imp β binding (IBB) domain of importin α (Imp α), which removes the autoinhibitory segment from the NLS-binding site.
2) Recognition of the NLS by the Imp α/β heterodimer whereby Imp α binds the NLS directly.
3) Docking of the Impα/β-NLS complex to the cytoplasmic face of the NPC through interaction of Imp β with Nups.

4) Translocation of the import substrate from the cytoplasmic side of the NPC to the nucleoplasmic side through a series of docking events to different Nups within the NPC.
5) Dissociation of the import substrate and Imp α from Imp β through binding of the guanine nucleotide binding protein Ran in a GTP bound form (Ran-GTP) to Imp β.

Through an analogous translocation mechanism occurring in parallel to the above series of events, nuclear transport factor-2 (NTF2) transports Ran-GDP to the nucleus where it is converted to Ran-GTP by the nucleotide exchanger RCC1, which is chromatin associated and hence concentrated in the nuclear compartment.

NLS recognition is by Imp α (Adam and Adam, 1994; Adam and Gerace, 1991; Radu *et al.,* 1995a), which comprises an NLS-binding domain consisting of ten Armadillo repeats (Conti *et al.,* 1998; Fontes *et al.,* 2000; Kobe, 1999), and an Imp β-binding (IBB) domain at the amino terminus (Görlich *et al.,* 1996a; Weis *et al.,* 1996). High affinity binding, to the order of 2-40 nM, of Imp α/β to NLS regions has been shown *in vitro* (Catimel *et al.,* 2001; Fanara *et al.,* 2000; Hu and Jans, 1999). In higher eukaryotes such as humans and mouse, there are multiple isoforms of Imp α (6 in humans) that appear to have distinct binding specificities for different NLS-containing substrates (Sekimoto *et al.,* 1997; Jans *et al.,* 2000b).

Docking of the importin-transport complex at the NE is mediated by interactions of Imp β with members of a family of soluble Nups associated with the NPC. These mobile Nups contain tandem sequence repeats (FxFG and GLFG) that are based on cores containing Phe and Gly (Rout *et al.,* 2000; Wente and Blobel, 1994) that are separated by variable lengths of hydrophilic residues (Buss *et al.,* 1994; Katahira *et al.,* 1999; Kehlenbach *et al.,* 1999; Radu *et al.,* 1995b). Nup-specific antibodies have been shown to inhibit nuclear transport (Clever and Kasamatsu, 1991; Featherstone *et al.,* 1988; Powers *et al.,* 1997), as does the lectin wheat germ agglutin (WGA), which inhibits transport through its ability to bind O-linked glycosidic N-acetyl-glucosamine moieties on FxFG Nups (Dabauvalle *et al.,* 1988; Hanover *et al.,* 1987). The essential role of these proteins in nuclear trafficking has also been shown by the fact that depletion of WGA-binding Nups inhibits transport, whilst re-addition of the WGA-binding protein

fraction restores transport activity (Finlay and Forbes, 1990).

Detailed interactions between Imp β and Nups have been shown *in vitro* (Chi and Adam, 1997; Radu *et al.,* 1995ab; Rexach and Blobel, 1995; Shah and Forbes, 1998) and *in vivo* (Damelin and Silver, 2000). It is likely that the initial docking step represents an interaction between nup358, a component of the cytoplasmic fibrils, and Imp β (Yaseen and Blobel, 1999ab; Yokoyama *et al.,* 1995). In contrast, interactions at the nuclear basket might represent the last step of import or the first step of export. Nup153 and Tpr are two components of the nuclear basket ring and intranuclear fibers, respectively. Whilst the Imp α/β/cargo complex seems to bind Nup153, binding of Imp β1 to Tpr apparently occurs only in the absence of cargo (Shah *et al.,* 1998; Balasundaram *et al.,* 1999). For the Imp α/β complex, these interactions may reflect the terminal step of protein import and the first steps of Imp recycling. Once the nuclear transport complex has been transported to the nuclear site, the Imp α/β/cargo complex is dissociated through binding of Ran-GTP by Imp β. Ran-GTP binding induces a conformational change in Imp β, causing the release of Imp α and the protein cargo. Subsequently, Imp β in complex with Ran-GTP is recycled back into the cytoplasm (Görlich and Kutay, 1999), whereas free Imp α requires the Imp β homologue (see Section 1.2.4) CAS for its export into the cytoplasm to be available for a new cycle of nuclear import (Kutay, 1997).

1.2.4 Importin β homologue-mediated nuclear protein import

While conventional nuclear protein import can be dissected into the above series of cellular events, import pathways have been identified that do not require Imp α for NLS recognition, with Imp β (Imp β1) or one of the many Imp β homologues (14 in yeast) mediating NLS recognition directly as well as mediating NPC/Nup docking and interaction with RanGTP as per its role in importin α/β-mediated nuclear import as illustrated in Fig. 1.7. The human immunodeficiency virus (HIV) protein Rev, human T-cell leukemia virus (HTLV-1) protein Rex, and the parathyroid hormone-related protein (PTHrP) all appear to be recognized by Impβ itself, independently of Impα (Truant and Cullen, 1999; Palmeri and Malim, 1999; Lam *et al.,* 1999). Many transcriptions factors such as AP-1, CREB, Fos and Jun, SRY and TRF1, which are constitutively localized in the nucleus, are transported into the nucleus in a similar, Impα independent fashion

(Forwood, 2001).) The shuttling heterogenous nuclear ribonucleoprotein (hnRNP) A1 protein possesses a 38-amino acid stretch, called the M9 region, which mediates nuclear import as well as nuclear export (Michael et al. 1995; Siomi and Dreyfuss 1995). Nuclear import of hnRNP A1 occurs via the M9 receptor transportin (Pollard et al. 1996; Siomi et al. 1997). In mammalian cells nuclear import of ribosomal proteins is mediated by the importin β-like proteins RanBP5, RanBP7, transportin, and the importin β subunit itself (Jäkel and Görlich 1998).

1.2.5 Importin/Ran-independent nuclear import

In addition to conventional importin/Ran mediated, NLS-dependent protein import, alternative pathways into the nucleus have been described (Efthymiadis *et al.,* 1998; Fagotto *et al.,* 1999; Michael *et al.,* 1997; Schmalz *et al.,* 1998; Sweitzer and Hanover, 1996). The HIV-1 Tat transactivator protein, for example, is not recognized by either importin α or β , but its NLS (G**RKKRRQRRR**AP^{59}; Siomi *et al.,* 1990) is sufficient to target the 476 kDa heterologous protein β-galactosidase into the nucleus in ATP-dependent but cytosolic factor-independent fashion (Efthymiadis *et al.,* 1998). Binding to components within the nucleus is mediated by the NLS. Another protein able to localise in the nucleus in the absence of importins is the multifunctional signalling protein β-catenin, a member of the Armadillo protein family, which itself is able to dock at the NPC (Fagotto *et al.,* 1999). In this case, β-catenin is able to bind to nucleoporins of the NPC directly. Like the Tat NLS, K-NLS (YD**RR**G**R**PGC**R**YDGMVGFSADET WDSAIDTWSPSEWQMAY^{361}) of the mRNA binding hnRNP K shuttling protein is not recognized by importins, and is able to mediate nuclear import in cytosolic factor-independent, but ATP-dependent fashion (Michael *et al.,* 1997). The NLS of the interferon-induced transcription factor IFI 16 (Q**KRKK**ST**K**E**K**AGPKGSKVSRDW^{145}) also mediates nuclear import in ATP-dependent, cytosolic factor-independent manner (Briggs *et al.,* 2001). The IFI 16 NLS is not recognized by importin α/β with high affinity and, like the Tat NLS, the IFI 16 NLS mediates binding to nuclear components, although, unlike Tat, this is modulated by protein kinase CK2. The 96 amino acid human immunodeficiency virus (HIV) protein Vpr does not have clearly identifiable modular NLSs (Jenkins *et al.,* 1998; Jans *et al.,* 2000); its nuclear import does not

require Ran-mediated GTP hydrolysis or ATP (Jenkins *et al.,* 1998). Jans *et al.* (2000) showed that intranuclear binding of Vpr depends on the presence of cytosolic factors, but conventional nuclear transport proteins such as importin α/ β and Ran are not responsible.

Schmalz *et al.* (1998) have shown that nuclear import of PKCα is independent of importin β as well as GTP. Since nuclear import cannot be inhibited by WGA, it appears to occur by a mechanism distinct from the one used by conventional NLS-containing proteins. (Beckmann *et al.,* 1994; Schmalz *et al.,* 1998). 20S proteasomes have no known NLS and are imported into the nucleus in an importin α/β-independent manner (Mayr *et al.,* 1999). Recently, a novel nuclear transport pathway for the spliceosomal proteins U1A and U2B has been described, which is ATP-dependent but requires neither cytosolic factors nor Ran (Hetzer and Mattaj, 2000).

Thus, it is clear that importin/Ran independent pathways exist for nuclear transport. The cellular factors responsible for mediating nuclear import in these cases remain uncharacterised, although the NLS sequences appear to have been defined.

1.3 Nuclear Import of growth factors, cytokines and their receptor systems

1.3.1 Introduction

Over the past 3 decades many different laboratories have demonstrated that polypeptide hormones and growth factors can associate with the nuclei of target cells, implicating the existence of an alternative transport pathway, to deliver endocytosed polypeptide to the nucleus instead of to lysosomes and degradation. In the following sections, the current state of knowledge about nuclear transport of polypeptide growth factors, hormones and their receptors is summarized.

1.3.2 Discovery

The first report of an exogenous polypeptide hormone being localized intracellularly was published by Stein and Gross (1959), who found that ^{125}I-labelled insulin localized in the cell interior after injection into rats. For the first time it was shown that intracellular translocation of an endogenously added growth factor can take place under physiological conditions, suggesting a direct involvement of polypeptide hormones at intracellular level in the regulation of cellular functions. In 1971, Angiotensin II was noted to be localized in the nuclear zone of vascular and cardiac muscle cells after being injected in the left ventricle of adult rats (Robertson and Khairallah, 1971). Other early examples of growth factors being localized in the nucleus of cell lines were Lactoferrin (Green *et al.*, 1971) and Nerve Growth Factor (NGF) (Andres *et al.*, 1977).

1.3.3 Nuclear import of growth factors/receptors

Nuclear association of polypeptide growth factors (GFs) and cytokines has been observed for a broad variety of target cells (Table 1.5). Although the idea of nuclear GFs is more or less accepted, the exact pathways involved in nuclear localization are far from fully understood and their functional significance is still generally debated. This section will summarize the experimental evidence for some of the growth factors and their receptors for which nuclear targeting has been described. Nuclear transport is juxtaposed with the role of the respective ligands in the better-accepted, conventional signalling pathways.

Table 1.5. Nuclear accumulation of polypeptide ligands and their receptors subsequent to receptor-mediated endocytosis

Polypeptide ligand	Nuclear accumulation of ligand after endocytosis	Nuclear binding sites for ligand	Nuclear accumulation of receptor after endocytosis	References
Amphiregulin	Ovarian epithelial carcinoma cells			Johnson et al. (1991) Lysiak et al. (1995)
Angiogenin	Pulmonary artery endothelial cells	Nucleoli		Moroianu and Riordan (1994b)
Angiotensin II	Smooth and cardiac muscle rat liver	Chromatin (liver, thymus)	Brain neurons	Robertson and Khairallah (1971); Tang et al. (1992); Jimenez et al. (1994); Lu et al (1998a, b); Chen et al. (2000); Peng et al. (2001)
CNTF	rat cortical type I astrocytes, glioma C6 cells, transfected COS-7 cells, Xenopus oocytes			Bajetto et al. (1999, 2000)
CTGF	Human mesangial cells			Wahab (2001)
EGF	Pituitary, colon carcinoma, vascular endothelial cells, regenerating liver, hepatocytes	Chromatin (colonic carcinoma)	Colonic carcinoma, pituitary cells, liver, corneal endothelial and granulosa cells	Raper et al. (1987); Rakowicz-Szulczynska et al. (1989); Jiang and Schindler (1990); Sibon et al. (1994) Lin et al. (2001)
FGF-1	Fibroblasts, vascular endothelial cells		Kidney, fibroblasts, pregnant rat uterus, hepatoma, adrenal medul-lary cells, myoblasts, astrocytes (FGF-R1α)	Cao et al. (1993) Wiedlocha et al. (1994, 1995, 1996)

Polypeptide ligand	Nuclear accumulation of ligand after endocytosis	Nuclear binding sites for ligand	Nuclear accumulation of receptor after endocytosis	References
GH	Liver, cardiovascular tissues, transfected CHO cells	Nucleoplasm, and inner and outer nuclear membranes, as well as chromatin (transfected CHO cells)	Adenohypophyseal cells, kidney, respiratory tissue, transfected CHO cells, cardiovascular tissues (GHR and GHBP)	Rezvani et al. (1973) Lobie et al. (1994 a, b) Waters et al. (1994)
HDGF	Vascular smooth muscle cells			Everett et al. 2001
IFN-β	Fibroblasts	Nucleus (fibroblasts)	Fibroblasts	Kushnaryov et al. (1986)
IFN-γ	Fibroblasts	Nuclear membrane (fibroblasts)	Fibroblasts	Bader and Weitzerbin (1994) MacDonald et al. (1986)
IL-1-α	Brown adipose tissue, fibroblasts		Brown adipose tissue	Grenfell et al. (1989); Wessendorf et al. (1993)
IL-1-β	T cells, brown adipose tissue		T cells, brown adipose tissue	Grenfell et al. (1991)
IL-2	Melanoma cells, lymphocytes			McMillan et al. (1995)
Insulin	Lymphocytes, adipocytes, fibroblasts, hepatoma cells	Nuclear membrane (liver, thyroid) Heterochromatin/nuclear membrane	Hepatocytes	Goldfine et al. (1977, 1985); Podlecki et al. (1987); Smith and Jarett (1987); Csaba and Hegyesi (1994)
LEDGF	Mouse lens epithelial cells			Fatma et al. (2000)
PDGF	SSV-transformed fibroblasts			Rakowicz-Szulczynska et al. (1986b); Yeh et al. (1987); Lee et al. (1987)
PTHrP	Cultured tumor cells, vascular smooth muscle cells		Antral follicles and oocytes, hepatocytes, granulosa cells	Nguyen and Karaplis (1998); Watson et al. (2000); Lam et al. (2000); de Miguel et al. (2001)

Polypeptide ligand	Nuclear accumulation of ligand after endocytosis	Nuclear binding sites for ligand	Nuclear accumulation of receptor after endocytosis	References
NGF	Pheochromocytoma, melanoma, rectal carcinoma cells	Chromatin (dorsal root neuron, colonic carcinoma), Nuclear membrane (pheochromocytoma)	Human melanoma cells	Yankner and Shooter (1979); Rohrer et al. (1982); Burwen and Jones (1987); Rakowicz-Szulczynska et al. (1986a);
NOVH	143 and HeLa cell line			Perbal, (1999)
Proenkephalin	Fibroblasts			Böttger and Spruce (1995)
Prolactin	Mammotrophs, lymphoma cells	Chromatin (T-cell lymphoma, adeno-hypophyseal tissue)	Lymphoma cells	Clevenger et al. (1990b); Rao et al. (1995a); Rao et al. (1995b) Clevenger et al. (2000) Rycyzyn et al. (2000)
Prothymosin α	HeLa, L929, IT-45R1, NIH3T3 cells, mouse thymocytes, human thymic stromal cells			Palvimo and Linnala-Kankkunen (1990); Manrow et al. (1991); Castro and Barcia (1996); Rubtsov et al. (1997)
SDGF	Swiss mouse 3T3 fibroblasts			Kimura et al. (1993)
VEGF	Endothelial cells			Li and Keller (2000)

CHO, chinese hamster ovary cells; CNTF, ciliary neurotrophic factor; CTGF, connective tissue growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; GH, growth hormone; HDGF, hepatoma-derived growth factor; IFN, interferon; IGFBP, insulin-like growth factor-binding proteins; LEDGF, lens epithelium-derived growth factor; NGF, nerve growth factor; PDGF, platelet derived growth factor; PTHrP, Parathyroid hormone-related protein; SDGF, schwannoma-derived growth factor; VEGF, vascular endothelial growth factor

The EGF receptor and its ligands EGF and SDGF

Epidermal growth factor (EGF) is a potent mitogen for epidermal cells *in vivo* and for a wide variety of cells in culture (Johnson *et al.,* 1980). EGF is a 5 kDa MW polypeptide produced by the submaxillary glands of the mouse. The prevailing mechanism by which EGF exerts its proliferative action is thought to be through the activation of mitogenic pathways such as the Ras/MAPK cascade. The EGF receptor, together with receptors for NGF, PDGF, FGF or insulin, belongs to the receptor tyrosine kinase family (RTK), whose members dimerize after ligand binding and undergo activation by autophosphorylation at multiple tyrosine residues in their cytoplasmic tails. The phosphorylated residues become docking sites for cytosolic and membrane-associated effector proteins containing SH2 domains, followed by the formation of second messengers and activation of the Ras/MAPK, PLC and PI3-K pathways (Boonstra *et al.,* 1995). However, it has long been known that several functions of the EGF/R system require mechanisms besides those activating early transient responses (Defize *et al.,* 1986; Wakshull *et al.,* 1985).

EGF as well as its receptor have been observed in the nucleus of a whole range of cell lines, human placenta, regenerating liver, human thyroid and many different cancer types (Johnson *et al.,* 1980; Savion *et al.,* 1981; Burwen *et al.,* 1984; Rakowicz-Szulczynska *et al.,* 1986b; Green *et al.,* 1987; Raper *et al.,* 1987; Marti *et al.,* 2001). The first demonstrations of the accumulation of EGF within cell nuclei were performed using cell fractionation as well as autoradiography, but seemed to require the use of the chemical chloroquine to inhibit lysosomal degradation of endocytosed EGF in cultured cells. In regenerating rat livers however, nuclear localization appeared to be physiological (Johnson *et al.,* 1980; Savion *et al.,* 1981). In normal rat livers, EGF was primarily transported to lysosomes and underwent degradation, while in regenerating liver cells, a significant part of intact EGF was transported into the hepatocyte nuclei instead (Raper *et al.,* 1987). A small percentage of the nuclear EGF was found to be transported to the nucleus together with the EGF receptor (Rakowicz-Szulczynska *et al.,* 1989). Nuclear transport of EGF receptor can be detected as early as 1 min after EGF incubation of different cell lines under physiological conditions, with a peak detected between 15 to 30 min (Lin *et al.,* 2001).

A common feature in the results of different experimental procedures identifying the nuclear EGF and its receptor was the strong correlation between nuclear localization and the highly proliferative status of tissues. A recent report provides the first evidence

that the EGF receptor might have a potential role as a transcription factor, suggesting that activated (i.e. phosphorylated) receptor molecules themselves can enhance gene expression by binding to regulatory domains of genes required for mitogenic effects, such as genes involved in the regulation of the cell cycle (Lin *et al.,* 2001). It was shown that the EGF receptor bound and activated AT-rich consensus-sequence-dependent transcription, including the consensus site of the Cyclin D1 promoter region. It was further demonstrated that nuclear EGF receptor was associated with the promoter region of Cyclin D1 *in vivo*. This observation is supported by previous reports that the EGF receptor can bind to chromatin after nuclear localization in target cells (Rakowicz-Szulczynska *et al.,* 1989). Of related interest is the observation that Schwannoma-derived growth factor (SDGF), also a ligand for the EGF receptor, requires nuclear localization to induce a mitogenic response in target cells (Kimura *et al.,* 1993). Additionally, it can bind to AT-rich DNA sequences that match the AT-rich minimal consensus sequences recognized by the EGF receptor. At present, it is not known how EGF or the EGF receptor are able to escape from the lysosomal degradation pathway. The mechanisms by which they are translocated to the nucleus also remain uncertain.

Fibroblast growth factor family (FGFs)

Fibroblast growth factors (FGFs) are pleiotropic growth factors that control cell proliferation, migration and differentiation. Many of the actions of FGFs have been demonstrated to be mediated through activation of high affinity cell surface receptors with intrinsic tyrosine kinase activity, ultimately activating different pathways, such as MAP kinase and ERK signalling cascades (Boilly *et al.,* 2000).

The prototypical ligand is basic FGF (FGF-2), a multifunctional cytokine involved in the proliferation and differentiation of a broad variety of mesodermal and neuro-ectodermal cell types (Bikfalvi *et al.,* 1997). Five different isoforms have been characterized, representing alternative translation products from a single mRNA (for review see Delrieu, 2000). The non-secreted, nuclearly targeted high molecular weight isoforms of FGF-2 (21.5, 22, 24, 34 kDa) have an intracrine mode of action, probably interacting with intracellular partners in the cytoplasm and/or the nucleus, and regulate the expression of specific target genes (Nakanishi *et al.,* 1992; Delrieu *et al.,* 1998). In contrast, the 18 kDa isoform, despite the lack of a classical signal peptide, is exported out of the cell through an endoplasmic reticulum/Golgi apparatus independent exocytosis pathway (Florkiewicz and Sommer, 1989). When secreted at the cell surface,

the 18 kDa FGF-2 can bind to heparan sulfate proteoglycan (HSPG) low affinity receptors and the high affinity receptor FGFR to act in an autocrine or paracrine manner (Florkiewicz *et al.,* 1998; Filla *et al.,* 1998). Following internalization, nuclear transport of 18 kDa FGF-2 has been observed in various cell types (Bouche *et al.,* 1987; Baldin *et al.,* 1990; Zhan et al. 1992; Hawker and Granger, 1994). In coronary venular endothelial cells, up to 50% of total internalized FGF-2 is rapidly targeted to the nucleus, suggesting that some biological activities of the growth factor may be mediated by nuclear FGF-2, subsequent to its binding to the cell surface receptors (Hawker and Granger, 1994). The nucleolar localization of 18 kDa FGF-2 is indeed correlated with the stimulation of ribosomal gene transcription, whose activation is mediated via the direct interaction of nuclear FGF-2 with the regulatory subunit of the protein kinase CK2 (Bonnet *et al.,* 1996). It is important to note that the 18 kDa FGF-2 isoform does not possess the NLS present in the NH2-terminal extension of the high molecular weight FGF-2 forms (Quarto *et al.,* 1991, and see below).

To date, five genes are known to encode for FGF receptors, with multiple variants due to alternative splicing (Powers *et al.,* 2000). One of the receptors, FGF-1, has been demonstrated to translocate to the nucleus of several cell types after stimulation with FGF-2 (Maher *et al.,* 1996; Stachowiak *et al.,* 1996ab). When the subcellular location of FGF-2 and its high affinity receptor FGFR-1 in the nervous system were investigated, it was shown that following the transition of cells to a subconfluent proliferating state, FGFR-1 translocated in parallel with FGF-2 to the nucleus, where both accumulated and acted to stimulate transition from the G0/G1 to the S phase of the cell cycle (Stachowiak *et al.,* 1997ab).

Prolactin and Growth hormone

Prolactin (PRL) and growth hormone (GH) are members of the somatolactogenic hormone family, the pleiotropic actions of which mediate vertebrate growth and mammary differentiation. The main functions of PRL and GH are thought to be mediated through binding to their respective receptors. The receptors of both are members of the same cytokine receptor type I family as that for IL-3/IL-5/GM-CSF (Goffin and Kelly, 1996). A characteristic of this receptor family is the lack of intrinsic tyrosine kinase activity (see Section 1.1.6). Instead, these receptors possess short homologous, proline-rich motifs involved in the direct binding of JAK protein kinases (Ihle *et al.,* 1997; Leonard and Lin, 2000).

None of the known signalling events triggered by PRL or GH are uniquely associated with any of the receptors. Indeed, the signalling mechanisms leading to specific function of PRL and GH have remained uncertain (Rycyzyn et al. 2000). It has been known for a number of years that the activation of PRL receptor-associated signalling pathways is linked to PRL internalization (Clevenger *et al.,* 1990a). PRL is internalized via an endosomal-like pathway, and transported across the ER and NE in an active process requiring co-stimulation by additional ligands, such as IL-2 in lymphocytes and EGF in breast epithelium (Clevenger *et al.,* 1990b). Nuclear translocation of PRL is of functional consequence, as nuclear PRL provides a necessary co-mitogenic stimulus for IL2- driven growth (Clevenger *et al.,* 1991). In addition, prolactin receptors have been detected in association with the NE (Clevenger *et al.,* 1990a). It has been proposed that PKC is required for nuclear targeting of PRL (Rao *et al.,* 1995). Recently, interaction of PRL with the peptidyl prolyl isomerase cyclophilin B (CypB) was revealed *in vitro* and *in vivo*. Experimental data indicated that CypB could serve as a chaperone, possibly mediating the retrotransport of PRL by CypB association with the ER sec61 transporter apparatus, and allowing nuclear transport of the PRL/CypB complex due to a putative N-terminal NLS in CypB (Rycyzyn *et al.,* 2000).

Early immunohistochemical studies have established that GH, GH receptor as well as the soluble GH receptor-binding protein (GHBP) are associated with the nucleus of a variety of target cells (Lobie *et al.,* 1990, 1994b). Approximately 20% of GH added to GH receptor expressing cells were found to be translocated to the nucleus (Lobie *et al.,* 1994a). It has been shown that rapid nuclear localization of GH receptor is critically dependent on serum starvation. Additionally, using a yeast two hybrid assay, a SYT (putative proto-oncogene involved in the transformed phenotype of synovial sarcomas) interacting protein (SIP), which possesses a putative NLS, was identified as potential GH receptor interacting protein, which may mediate nuclear localization of the GH receptor (Waters *et al.,* 2001). The physiological role of nuclear localized GH receptor and ligand is not yet understood, but the above-mentioned results indicate that nuclear localization of the GH receptor may be important in modulating cell sensitivity to the proliferative action of GH.

1.3.4 Nuclear localization sequences in polypeptide hormones and growth factors

The exact means by which polypeptide ligands, growth factor and their respective receptors are translocated to the nucleus after internalization are not well understood. What is striking though is the existence of regions containing basic amino acid residues resembling NLSs in many of the nuclear localized ligands (Table 1.6).

Evidence for an important role of NLSs in signalling arises from the fact that multiple forms of the same ligand, produced by alternative splicing or through the use of multiple in-frame start codons, can exhibit different physiological effects. For example, the NLS-containing forms of FGF-2 and PDGF-A are more potent in mitogenic signalling than their NLS-lacking counterparts (Maher et al. 1989; Quarto *et al.,* 1991; Rifkin *et al.,* 1994). A good example of the importance of an NLS for full functionality is that within FGF-1. Induction of mitogenesis by FGF-1α and FGF–1β appears to be directly dependent on nuclear localization (Imamura *et al.,* 1990). Deletion of the N-terminal NLS abolished stimulation of proliferation without interfering with other functions, whilst full mitogenic activity could be restored by the inclusion of the histone H2B NLS, demonstrating that nuclear targeting is integral to the biological function of FGF-1 (Imamura *et al.,* 1990).

Basic regions, resembling NLSs, have also been noted in IL-5 (Fig.1.8). In particular, these regions carry the feature of a bipartite NLS (see Section 1.2.4) with two of the three basic segments, separated by a spacer of 12 amino acid residues (Jans *et al.,* 1997a; see Section 1.4). Mutational analysis of this NLS and its role in IL-5 function is in part the subject of Chapter 3.

Table 1.6 Overview of polypeptide ligands and growth factors containing putative or confirmed NLSs.

Protein	Species	Putative NLS	Reference
Amphiregulin	human	KPKRKKKggkngknRRNKKK[43]	Johnson et al. (1991) Modrell et al. (1992)
Angiogenin	human	RRRGL[35 a]	Moroianu and Riordan (1994ab); Lixin et al. (2001)
CTGF	human	PFPRRVK[155]	Wahab et al. (2001)
FGF-1 (aFGF)	human	RQKRQAR-10 a.a. spacer- KRRSSPSKDGR[38] RKRPVRRR[65]	Shiurba et al. (1991) Cao et al. (1993)
FGF-3	mouse	RLRRdaggrggvyehlggapPRRRK[76/47 b]	Kiefer et al. (1994) Kiefer and Dickson (1995) Antoine et al. (1997)
IFN-γ	human	NSNKKKR[92] KTGKRKRS[135]	Bader and Weitzerbin (1994) Subramaniam et al. (1999)
IL-1 α	human	KVLKKRRL[86]	Grenfell et al. (1989) Wessendorf et al. (1993)
IL-1 β	human	PKKKMEKRF[99]	Grenfell et al. (1991)
LEDGF	human	RRGRKRK[152]	Fatma et al. (2000)
NOVH	human	KKGKKCLRTKKS	Perbal (1999)
PDGF A	human	PRESGKKRKRKR[207]	Collins et al. (1987)
PDGF B	human	RVTIRTVRVRRPPKGKHRK[223/255]	Lee et al. (1987)
Proenkephalin	human	KRYGGFMRGLKR[194]	Böttger and Spruce (1995)
Prothymosin α	human	TKKQKT[107 c]	Palvimo and Linnala-Kankkunen (1990); Manrow et al. (1991) Rubtsov et al. (1997) Shakulov et al. (2000)
PTHrP	human	KTPKKKK[93 d]	Henderson et al. (1995) Lam et al. (1999; 2001)
SDGF	human	KRKKK[119] 10 a.a. spacer RKKKK[134]	Kimura (1993)

CTGF, connective tissue growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; IFN, interferon; LEDGF, lens epithelium-derived growth factor; PDGF, platelet derived growth factor; PTHrP, parathyroid hormone-related protein; SDGF, schwannoma-derived growth factor.

[a] not recognized by Imp α or β ; [b] Imp α/β recognized; [c] NLS is recognized by yeast Imp α (Srp1p), but Imp α recognition is not sufficient for nuclear targeting; [d] Imp β mediated.

It has been proposed that ligands localized in the nucleus by NLS-dependent pathways may influence transcription

1) through binding to chromatin or DNA;
2) by activating nuclear kinases or other nuclear enzymes;
3) through association with transcription factors; or
4) by co-targeting their receptor subunits to the nucleus.

FGF-2 has been demonstrated to bind histone H1 with high affinity (Bouche *et al.*, 1987), and may be involved in activating rDNA transcription through binding to specific sequences in the non-transcribed spacer of ribosomal genes (Amalric *et al.*, 1994). FGF-2 has also been shown to interact with the protein kinase CK2, and can directly modulate CK2 activity toward nucleolin, a primary CK2 substrate *in vitro* (Bonnet *et al.*, 1996), so that it has been postulated that FGF-2 may target CK2 to the nucleolus to modulate rDNA transcription.

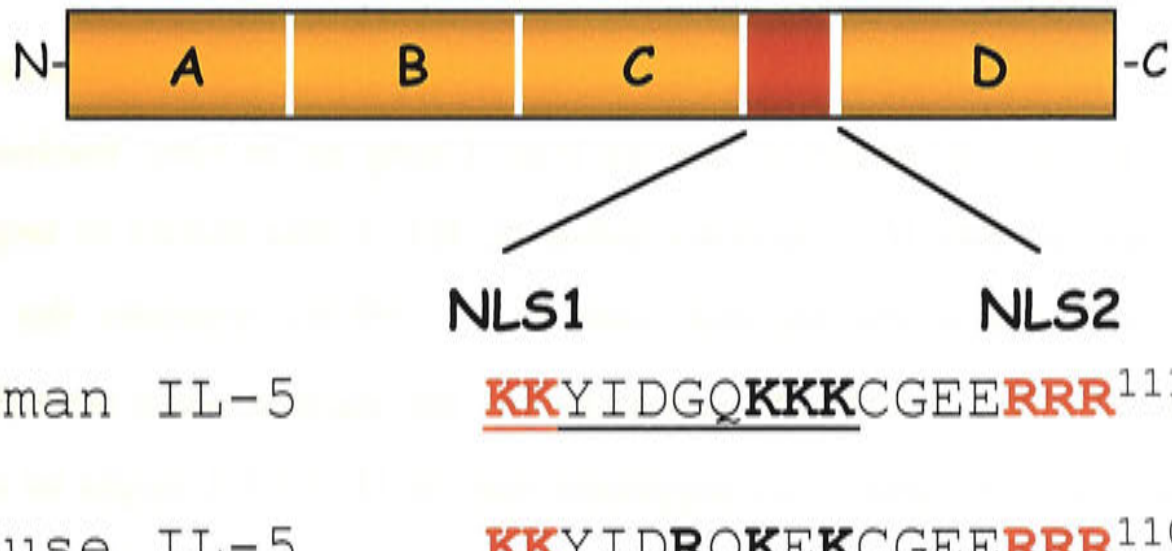
Ligands such as insulin and PDGF can associate with chromatin, implying that these ligands could modulate chromatin structure and thus gene expression (Jans, 1994).

An additional function of NLS-dependent nuclear targeting of polypeptide ligands may be to co-target their associated receptors to the nucleus (Jans, 1994; Jans and Hassan, 1997). Once in the nucleus, the ligand-receptor-complex or the receptor on its own could influence transcription in similar fashion to the proposed action of ligands (see above). For example, the NLS-containing forms of FGF-1 and FGF-2 co-target the FGF-1 receptor, a tyrosine kinase, to the nucleus in a functional active form, where it may activate nuclear transcription factors by phosphorylation (Maher, 1996; Prudovsky *et al.*, 1994; Stachowiak *et al.*, 1996a). As mentioned earlier, it was shown recently that the EGF receptor can bind and activate AT-rich consensus-sequence-dependent transcription, including the consensus site of the Cyclin D1 promoter region (Lin *et al.*, 2001). Additionally, the GH receptor was shown to activate JAK2 and PLC leading to transcriptional activation of the TF c/EBP (Waters *et al.*, 1994), whilst the GH-GHBP complex appears to activate PKC (Lobie 1991).

The exact mechanisms of how nuclear transport of polypeptide ligands takes place are mostly unknown. For a number of polypeptide ligands, recognition by importins (see Section 1.2.3) has been reported (see footnote to Table 1.6), implying that these may be imported into the nucleus by conventional nuclear import pathways.

1.4 Nuclear Import by IL-5

The elucidation of the main pathways of IL-5 signalling have thus far not led to an explanation of its specific role in the differentiation of eosinophils, since IL-3 and GM-CSF activate the same signalling cascades revealed so far (see Section 1.1.9). Specificity may therefore either lie with the IL-5 receptor α subunit or in the IL-5 ligand itself. In fact, basic regions, resembling NLSs, have been noted in both human and mouse IL-5 (Fig. 1.8).



human IL-5	KKYIDGQKKKCGEERRR[111]
mouse IL-5	KKYIDRQKEKCGEERRR[110]
SWI5	KKYENVVIKRSPRKRGRPRK[655]
N1/N2	RKKRKTEEESPLKDKDAKKSK[554]
Nucleoplasmin	KRPAATKKAGQAKKKLDK[174]
Consensus	KK -10-12 aa- KKK RR RRR

Fig. 1.8. The NLSs of human and mouse IL-5 compared to the bipartite NLSs of SWI5, N1/N2 and nucleoplasmin.
Amino acids are are shown in the single-letter code. The proximal and distal arm of the bipartite NLSs are in red. The sequence underlined represents the putative novel NLS of IL-5, with basic residues indicated in bold (for references, see Table 1.3B). aa, amino acid

In particular, these regions carry the feature of a bipartite NLS (see Section 1.2.4) with two of the three basic segments, separated by a spacer of 12 amino acid residues. Of the three ligands IL-3/IL-5 and GM-CSF, only IL-5 exhibits this feature.

The nuclear targeting capacity of fluorescently labelled human IL-5 has been visualized in intact, hIL-5-receptor-expressing FDCP1 mouse myeloid progenitor cells, using *in vivo* and *in vitro* nuclear transport assays (Jans *et al.,* 1997a). The hypothesis that two basic regions at the carboxy-terminus, called NLS1 and NLS2 (see Fig. 1.8), may be responsible for this nuclear targeting activity was tested using IL-5 NLS-containing β-galactosidase fusion proteins. The IL-5 NLS was demonstrated to target β-galactosidase to the nucleus in both microinjected cells and *in vitro* with mutation of the proximal arm of the bipartite NLS abolishing NLS function (Jans *et al.,* 1997a).

As mentioned in Section 1.3, several polypeptide ligands have been observed to co-translocate their surface receptors to the nucleus. Using an *in vitro* nuclear transport assay for hIL-5 and soluble IL-5 receptor subunits, hIL-5 was shown to target both α- and β- receptor subunits to the nucleus (Jans *et al.,* 1997b), whereby the non-ligand binding β receptor subunit could only accumulate in the nucleus when both IL-5 and the α receptor subunit were present. This suggested that the IL-5 NLS might be specifically required to enable the nuclear entry of the α and β receptor chains. The importance of the NLS1 and NLS2 clusters in piggy-backing of the IL-5 receptor subunits was tested by mutating either arm of the putative bipartite NLS, whereby mutation of the proximal arm abrogated IL-5's ability to co-target the receptor subunits to the nucleus (Calanni, 1997). Unexpectedly, mutations in the distal arm (NSL2) had no influence on the nuclear targeting of the receptor subunits, indicating that the NLS of IL-5 was not a classical bipartite NLS (Calanni, 1997).

1.5 Aims and scope of the research project

The above review of IL-5 signal transduction indicates that there are large gaps in our knowledge regarding the molecular basis of the specificity of signaling distinct to that mediated byIL-3 and GM-CSF. IL-5 clearly plays an important role in the pathogenesis of allergic diseases such as asthma. so that it is of considerable interest to identify the

signalling mechanisms specific to IL-5. The findings indicating nuclear translocation of receptor-internalized IL-5 (Jans *et al.,* 1997a) imply that the ligand may have a direct role in signal transduction in the nucleus. This capability would appear not to be possessed by IL-3 or GM-CSF, and conceivably represents the basis of the specific effects exhibits on the eosinophil lineage.

In this study, the mouse myeloid progenitor FDC-P1 cell line is used to study the pathway of nuclear transport of IL-5 and its receptors. In particular, the main aims are

1) To define the putative NLS of IL-5

2) To determine whether nuclear transport of IL-5 occurs via conventional nuclear import pathways, and

4) To investigate the transport of the IL-5 receptor to the nucleus *in vivo*.

Various IL-5 NLS-mutant and fusion protein derivatives were analyzed to ascertain the functionality and nature of the putative NLS and critical residues thereof. A well-established *in vitro* nuclear transport assay was employed in conjunction with CLSM and a previously developed ELISA-based binding assay (Efthymiadis *et al.,* 1997; Hübner et al. 1997, Xiao *et al.,* 1997) used to assess the recognition of IL-5 and its NLS by importins.

For receptor binding studies of various NLS mutant derivatives, a flow cytometry-based technique was developed. For the *in vivo* studies, a protein precipitation procedure based on biotinylation of IL-5 and subcellular fractionation of IL-5 receptor-expressing FDC-mIL-5R cells was used.

Through the use of the techniques and assay described above, this study demonstrates that IL-5 possesses a novel NLS mediating nuclear import through a pathway independent of importins and Ran. This NLS is presumably the basis of its ability to cotransport its α receptor subunit to the nucleus subsequent to receptor-mediated endocytosis. Thus, it can be hypothesized that receptor targeting to the nucleus is a key signalling event in IL-5 specific signal transduction that requires nuclear localizing activity on the part of IL-5.

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

All chemicals and enzymes used were of analytical grade or better.

2.1.2 Buffers, solutions and culture media

All buffers, solutions, and culture media were prepared with double-distilled water and sterilised by autoclaving at 121°C for 20 min. Media prepared for bacterial and cell culture, solutions for DNA manipulations, protein analysis and *in vitro* assay techniques are described in Table 2.1.

Table 2.1. Buffer, solutions and culture media used in this study (For abbreviations see page vii).

Use	Name of solutions	Composition	Additional Information
Bacterial Culture	Luria-Bertani (LB)	per litre: 10 g bacto-tryptone 5 g bacto-yeast extract 10 g NaCl	autoclaved; 20 mM glucose (solid) or glycerol (liquid) added antibiotics added
Cell Culture	PBS	137 mM NaCl 6.75 mM Na_2HPO_3 2.5 mM NaH_2PO_3	pH to 7.4 with HCl
	RPMI	RPMI Powder 10.44g/L 24 mM Sodium Bicarbonate	

	Media for FDC-P1 cell line	RPMI 10% FBS heat inactivated 10 mM NaCl 1mM Na Pyruvate 0.0006% Monothioglycerol Penicillin 60 mg/ml Streptomycin 100 mg/ml Gentamycin 10 mg/ml	
	Media for CTLL-2 cell line	RPMI 10 % FBS (heat inactivated) Penicillin 60 mg/ml Streptomycin 100 mg/ml Gentamycin 10 mg/ml 0.05 mM 2ME	
DNA manipulation	7x DNA loading buffer	70 mM Tris-HCl 88 mM EDTA 2.8 % Sarcosyl 0.7 mg/ml BPB 100 mg/ml Ficoll 400	
	TAE	0.04 M Tris-acetate 0.01 M EDTA, pH 8.0	
	TE	10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0	
	$T_{10}E_{0.1}$	10 mM Tris-HCl 0.1 mM EDTA	pH to 7.5 with HCl
Reverse transcription	5 x AMV-RT buffer	250 mM Tris-HCl, pH 8.3 250 mM KCl 50 mM MgCl2 50 mM DTT 2.5 mM Spermidine	
Protein expression, purification and detection	Coomassie Blue Staining Solution	0.25% Coomassie blue 45% Methanol 7% Acetic acid	
	Destaining Buffer	45% Methanol 7% Acetic acid	

	Electrophoresis Buffer	25 mM Tris-HCl 192 mM glycine 0.1% SDS	pH to 8.3 with HCl
	2x SDS Lysis Buffer (denaturing, reducing)	0.13 M Tris-HCl, pH 6.8 4% SDS 20% glycerol 0.005%BPB 5% β-Mercaptoethanol	
	6x SDS Lysis Buffer	0.13 M Tris-HCl, pH 6.8 4% SDS 60% glycerol 0.005% BPB 5 % β-Mercaptoethanol	
β-Gal Fusion Protein Purification	Protein Incubation Buffer	20 mM Tris-HCl, pH 7.4 10 mM MgCl2 10 mM β-Mercaptoethanol	
	Equilibration Buffer	20 mM Tris-HCl, pH 7.4 1.6 M NaCl 10 mM $MgCl_2$ 10 mM β-Mercaptoethanol	
	Elution Buffer	100 mM Boric Acid-NaOH, pH 10.1	
	10 x Injection Buffer	48 mM K_2HPO_4 14 mM NaH_2PO_4 45 mM KH_2PO_4	pH to 7.4
	Dialysis Buffer	20 mM Tris-HCl, pH 7.4 10 mM $MgCl_2$ 10 mM β-Mercaptoethanol	
Gel shift Assays	TBE	9 mM Tris-HCl 9 mM boric acid 0.2 mM EDTA	

	Gel shift binding buffer	10 mM Tris 1 mM $MgCl_2$ 0.1 % NP-40 10 mM DTT 0.8 mM EDTA 3 % glycerol 1.5 % sucrose	
Cell Fractionation	Buffer A^- ($NP\text{-}40^-$ lysis buffer)	0.34 M Sucrose 10 mM HEPES, pH 8.0 60 mM KCl 2 mM EDTA 0.5 mM EGTA	added freshly: 1.5 mM DTT 0.5 mM Spermine 0.15 mM Spermidine
	Buffer A^+ (NP-40 lysis buffer)	Buffer A^- 0.5% NP-40	
	Buffer C (Nuclei lysis buffer)	400 mM NaCl 7.5 mM MgCl2 0.2 mM EDTA 0.1 mM EGTA 1 mM DTT	added freshly: 0.5 mM PMSF 10 mg/ml Aprotinin 5 mg/ml Leupeptin
Whole Cell Lysis	RIPA buffer	50 mM Tris-HCl, pH 8.0 150 mM NaCl 0.2% SDS 0.5% DOC 0.5% NP-40	added freshly: 1 mM PMSF 0.15 units Aprotinin 10 mg/ml Leupeptin 1 mM Sodium orthovanadate
Western Blot	Transfer buffer	192 mM glycine 25 mM Tris-HCl pH 8.3 20 % methanol	
	Blocking buffer	PBS 5% (wt/vol) BSA 0.3 % Tween 20	
	Wash buffer (PBS-T)	PBS 0.3 % Tween 20	
	Antibody dilution buffer	PBS 0.3 % Tween 20 2% (wt/vol) BSA	

	Stripping buffer	67 mM Tris-HCl pH 6.7 10 mM DTT 2% SDS	
EM	Fixation Buffer	PBS, pH 7.3 0.1 M Sucrose 2-4% PFA 0.25% Glutaraldehyde	
	Wash buffer A (S-PBS)	PBS, pH 7.3 0.1 M Sucrose	
	Wash buffer B (SN-PBS)	PBS, pH 7.3 0.1 M Sucrose 50 mM NH_4Cl	
	Blocking buffer	PBS, pH 7.3 1% BSA 0.1% CWFG	
	Antibody dilution buffer	PBS, pH 7.3 1% BSA 0.5% Tween-20	
***In vitro* Nuclear Import Assay**	Intracellular Buffer (IB)	110 mM KCl 5 mM $NaHCO_3$ 5 mM $MgCl_2$ 1 mM EGTA 0.1 mM $CaCl_2$ 20 mM Hepes	pH to 7.4 with HCl autoclaved freshly added: 1 mM DTT 10 µg/ml leupeptin
	ATP regenerator	1 X IB 6.25 mg/ml creatine phosphokinase 500 mM creatine-phosphate 100 mM ATP	
ELISA	Blocking Buffer	PBS 10% (wt/vol.) skim milk powder	
	PBS/Tween/ BSA Solution	1% BSA 0.3% Tween 20	

	Hybridisation buffer	1 X IB 1 mM DTT 1% BSA	
	Diethanolamine buffer	10 % diethanolamine 0.5 M $MgCl_2$, pH 9.8	
Dot Blot	Assembly mix	13.6 µM final concentration heterodimer GST-importin α/β 1 x IB 1 mM DTT	

2.1.3 Kits

The molecular biology kits used in this study are listed in Table 2.2.

Table 2.2. Molecular biology kits used in this study.

Kit name	**Company**
U.S.E Mutagenesis Kit	Pharmacia Biotech
QIAEXII Gel Extraction Kit	QIAGEN
Plasmid Midi Kit	QIAGEN
Wizard Plus Mini Plasmid Prep Kit	Promega
SuperSignal West Pico Western Blot Kit	Pierce
ECL Plus Western Blot Kit	Amersham
AP detection kit	Pharmacia

2.1.4 Bacterial strains

The *Escherichia coli* bacterial strains used in this study are listed in Table 2.3.

Table 2.3. Bacterial strains used in this study.

DH5α	F-, ø80dlacZDM15, D(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, l-, thi-1, gyrA96, relA1
Top 10 F'	F' {lacI^{q}Tn1-(Tet^{R})} mcr A Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR recA1 araD139 D(ara-leu)7697 galU galK rpsL endA1 nupG
BL21 (DE3)	F^{-}, ompT, hsdSB(rB-mB-), gal, dcm (DE3)
TG1	F′, traD36, lacIq(lacZ)M15, proA+B+/supE (hsdM-mcrB)5 (rk-mk+ McrB-), thi (lac-proAB)
NM522 (MutS)	supE, thi-1, D(lac-proAB), D(mcrB-hsdSM)5, (rK-mK-), (F' proAB lacIq,D (lacZ)M15)
MC 1060	F^{-} hsdR (r_K^{-},m_K^{+}) araD139 D(araABC-leu)7679 galU galK DlacX74 rpsL thi mcrB}

2.1.5 Restriction endonucleases

Restriction endonucleases used in this study are listed in Table 2.4.

Table 2.4. Restriction endonucleases used in this study.

Restriction endonucleases	**Source**
Bal I	Promega
Bam H I	NEB
Cla I	Bresatec
Dpn I	Fermentas
Eco R I	NEB
Stu I	MBI

2.1.6 Cloning and expression vectors

Plasmids used in this study are listed in Table 2.5.

Table 2.5. Plasmids used in this study.

Plasmid	Description	Encoded protein
pPR2hIL5	Contains a Tac promoter which directs the expression in bacteria of the *E. coli* β-galactosidase enzyme (coding for amino acids 9-1023), contains the wild type NLS region of human IL-5 (coding for amino acids 93-112), inserted into SmaI restriction endonuclease site of plasmid pPR2	hIL-5NLSwt β-gal
pPR2hIL5	Derived from pPR2hIL5, contains NLS mutant region of human IL-5 (coding for amino acids 93-112), mutated at K95T and K96T	hIL-5NLS1 β-gal
pPR2hNKA	Derived from pPR2hIL5, contains NLS mutant region of human IL-5 (coding for amino acids 93-112), mutated at K102N and K104A	hIL-5NLS3 β-gal
pPR2mKEK	Derived from pPR2hIL5, contains NLS mutant region of mouse IL-5 (coding for amino acids 92-111), mutated at G98R and K101E	mIL-5wt β-gal
pBacPak8hIL5scHSA	Contains a AcMNPV polyhedrin promoter which directs expression of the hIL-5 single chain HSA fusion protein in insect cells, cloned into the BamH1/EcoR1 site of pBacPAK8 baculovirus expression vector	hIL5scHSA
pcDEF3mIL5Rα	Contains an human EF-1alpha promoter which directs the expression of the mouse IL-5Ra chain in mammalian cells; Contains an SV40 promoter which directs the expression of the neomycin resistance gene that confers resistance to the antibiotic G418, and contains full length mIL-5 receptor alpha subunit cDNA cloned into the BamH1/Nco1 site of pcDEF3 expression vector	full length mIL5Rα

2.1.7 Oligonucleotides

Oligonucleotides used in this study are listed in Table 2.6.

Table 2.6. Oligonucleotides used in this study.

Mutagenesis Primers	**Primer Sequence**
IL-5NLS1 SENSE	5'-CTT GTC CTT AAT AAC GAC ATA CAT TGA CGG C-3'
IL-5NLS1 ANTI	5'-GCC GTC AAT GTA TGT CGT TAT TAA GGA CAA G-3'
hNLS3NKA	5'-TAC ATT GAT GGT CAA AAC AAG GCC TGT GGA GAA G-3'
NLS3 ATT	5'-ATT GAC GGC CAA AAC AAG GCC TGT GGA GAA GAA AG-3'
NLS3 COMP	5'-CTT TCT TCT CCA CAG GCC TTG TTT TGG CCG TCA AT-3'
mNLS wt	5'-GAA ATA CAT XGA TCG CCA AAA AGA AAA GTG TGG-3'
mKEK SENSE	5'-GAA ATA CAT TGA TCG CCA AAA AGA AAA GTG TGG AG-3'
mKEK ANTI	5'-CTC CAC ACT TTT CTT TTT GGC GAT CAA TGT ATT TC-3'
	5'-CGT CTT TCT TCT CCA CAC TTT TC3'
Sequencing Primers	**Primer Sequence**
pPR2SEQ1	5'ACT CCC GTT CTG GAT AAT GT3'
pPR2SEQ2	5'TTC TCC GTG GGA ACA AAC G3'
PCR Primers	**Primer Sequence**
hNLS1 VERIFY	5'GTT TTG GCC GTC AAT GTA TGT CG3'
NLS3 VERIFY	5' CTC CGT CTT TCT TCT CCA CAG G3'
mKEK VERIFY	5'CGT CTT TCT TCT CCA CAC TTT TC3'
pPR2HOT1	5'GGT CGT AAA TCA CTG CAT AAT TCG3'

2.1.8 Antibodies and Conjugates

The primary antibody used in this study are listed in Table 2.7.

Table 2.7. Primary antibodies used in this study.

Primary Antibody against	Type	Company
mouse **IL-5**	goat polyclonal (biotinylated)	R&D
human/mouse **IL-5**	rabbit polyclonal	Santa Cruz
mouse **IL-3 Receptor α** (C-terminus)	rabbit polyclonal	Santa Cruz
mouse **IL-5 Receptor α** (C-terminus)	rabbit polyclonal	Santa Cruz
mouse **GM-CSF Receptor α** (C-terminus)	rabbit polyclonal	Santa Cruz
mouse **Receptor β_c**	rabbit polyclonal	Santa Cruz
human **IL-5 Receptor α** (C-terminus)	rabbit polyclonal	Santa Cruz
human **Receptor β_c**	mouse monoclonal	Santa Cruz
human **Receptor β_c** (C-terminus)	rabbit polyclonal	Santa Cruz
human **Receptor β_c** (N-terminus)	rabbit polyclonal	Santa Cruz
human/mouse **importin β**	goat polyconal	Santa Cruz
mouse **IL-3**	goat polyconal	Santa Cruz

The secondary antibody conjugates used in this study are listed in Table 2.8.

Table 2.8. Secondary antibodies conjugates used in this study.

Secondary Antibody/Conjugates	Company
HRP conjugated anti rabbit Ig	Amersham
HRP conjugated anti mouse Ig	DAKO
HRP conjugated anti goat Ig	Pierce
Gold conjugated anti-rabbit IgG (10nm gold)	Amersham
HRP conjugated Streptavidin	Silenus
Streptavidin conjugated Dynabeads M-280	Dynal

2.1.9 Enzymes and markers

The enzymes and markers used in this study are listed in Table 2.9.

Table 2.9. Enzymes and markers used in this study.

Enzymes, markers	Application	Source
T4-DNA ligase	Ligation	Biolabs
native Pfu DNA Polymerase	PCR	Stratagene
Pfu turbo DNA Polymerase	PCR	Stratagene
Alkaline phosphatase (calf intestinal)	Dephosphorylation	Pharmacia
Taq polymerase	PCR verification	Fermentas/Invitrogen
GeneRuler 1kb DNA Ladder	DNA Agarose gels (500 kb to 10.000 kb)	Fermentas
pUC19 DNA/MSPI DNA Ladder	DNA Agarose gels (40 kb to 500 kb)	Fermentas
BenchMark Protein Ladder	SDS PAGE/ Coomassie Staining	Gibco BRL
BenchMark Protein Ladder/prestained	SDS PAGE/Western Blot	Gibco/Invitrogen

2.1.10 Miscellaneous materials

For Western Blot procedures, 3MM chromatography paper was used from Whatman Ltd., and nitrocellulose membrane was provided by Schleicher and Schuell.

2.2 Molecular Methods of DNA Manipulation

I. *E.COLI* MANIPULATION

2.2.1 Preparation of competent E. coli cells

Competent *E.coli* cells were prepared for DNA transformation using two different methods:

a) $CaCl_2$ method

E. coli cells were streaked out onto an agar plate and grown overnight at 37°C. A single colony was used to inoculate 3 ml of LB and incubated at 37°C overnight with agitation. 400 ml of LB was inoculated with 100 µl of the overnight culture and grown at 37°C to an OD_{600} of 0.8-1.0 with vigorous shaking on a Controlled Environment Incubator Shaker (New Brunswick Scientific Co. Inc. USA). To harvest the cells, the culture flasks were chilled on ice for 15 min, the cell suspension transferred into 500 ml centrifuge tubes and centrifuged in a cold GS-3 rotor (Sorvall RC5C, Sorvall Instruments, DuPont) at 4800 rpm for 15 min. After removing the supernatant the pellet was resuspended in 50 ml cold, sterile $CaCl_2$ (0.1 M) and incubated overnight at 4 °C. After spinning for 5 min at 5000 g, the supernatant was discarded and the pellet resuspended in 5 ml cold mix of 0.1 M $CaCl_2$ and glycerol (85:15). The cell suspension was aliquoted into pre-chilled Eppendorf tubes and stored at -70 °C until use.

b) Electrocompetent cells

Two times 400 ml of LB were each inoculated with 400 µl of a fresh overnight culture. The cells were grown at 37 °C to an OD_{600} of 0.7 with vigorous shaking on a Controlled Environment Incubator Shaker (New Brunswick Scientific Co. Inc. USA). To harvest the cells, the culture flasks were chilled on ice for 15 min, the cell suspension transferred into 500 ml centrifuge tubes and centrifuged in a cold GS-3 rotor (Sorvall RC5C, Sorvall Instruments, DuPont) at 4800 rpm for 15 min. After removing the supernatant, the pellet was carefully resuspended in 1 ml of ice-cold water, topped up with cold water to 1 L and centrifuged as above. This procedure was repeated with 500 ml and 20ml of ice-cold water. The cells were resuspended in a final volume of 3 ml cold 10% glycerol in water. The cells

were subsequently aliquoted into pre-chilled Eppendorf tubes, frozen on dry ice and stored at -70 °C until use.

2.2.2 Transformation of competent E. coli cells

Transformation of competent bacteria was performed using the heat shock procedure based on that of Sambrook et al. (1989). Plasmid (1 µg) or half of a ligation mix was mixed with competent cells (200 µl) and incubated on ice for 30 min before a 90 sec heat shock at 42°C. The culture was then placed on ice for 2 min followed by the addition of 1 ml of LB and incubated at 37°C for 1 h. For routine plasmid transformations, 200 µl of the transformation culture was plated onto an agar plate containing the requisite selective antibiotic(s), while in the case of ligations, 50 µl of the bacterial culture was plated, and the remaining culture centrifuged, resuspended in 50 µl of LB and additionally plated out on a separate plate. Plates were incubated at 37°C overnight and colonies picked and used to inoculate 5 ml of LB with antibiotic, incubated at 37°C overnight (shaking 200 rpm) to be used for DNA preparation and further analysis.

2.2.3 Long term storage of *E. coli* cells

E. coli strains containing various plasmids were grown up for storage in 5 ml cultures to late log phase (OD_{595} of 1.0). To 1 ml aliquots of culture was added 1ml of 30% sterile glycerol-LB mix and the cells gently inverted and then stored at -70°C.

II. PREPARATION AND ANALYSIS OF DNA

2.2.4 Plasmid preparation

Preparations of ~5 µg plasmid DNA were carried out using a Kit from Promega (see Table 2.2), according to the procedure recommended by the manufacturer. Briefly, 4 ml of bacteria grown at 37°C for 12 h (shaking at 200 rpm) under the selection of an appropriate antibiotic were collected by centrifugation (14.000 rpm, 1 min) and lysed using alkaline lysis buffer (included in kit). Following pH neutralization, the cell debris was removed by centrifugation (Eppendorf benchtop centrifuge, 14.000 rpm, 1 min) and the supernatant

passed through a spin filter. The solution was washed and eluted in 50 µl TE buffer. Plasmid amounts of approximately 100 µg were prepared using the QIAGEN Plasmid Midi Kit (see Table 2.2) using essentially the same method and buffers as above.

2.2.5 Agarose gel electrophoresis

Separation of DNA fragments of greater than 100 bp was performed by electrophoresis in 0.8-2.0% agarose gels in TAE buffer (see Table 2.1) using a horizontal slab gel apparatus (BioRad) run at 60-100 V for 2-4 h. Agarose gels were stained in 0.5 µg/ml ethidium bromide (EtBr) and DNA fragments visualized under fluorescent UV light.

2.2.6 Quantitation of DNA

DNA samples were quantitated spectrophotometrically based on absorbances at 260 nm. The concentration of DNA in plasmid preparations was calculated on the basis that an OD of 1 at 260 nm corresponds to 50 µg/ml of double stranded DNA.

2.2.7 Recovery from agarose gels

Recovery and purification of DNA fragments from agarose gels was performed by gel extraction according to the protocol given with the gel extraction kit (Qiagen). Following separation by agarose gel electrophoresis (see section 2.2.5), the band of interest was located under UV illumination using a Transilluminator (Gel Documentation System, Novaline), the band removed using a scalpel, placed into an eppendorf tube and the DNA recovered using a gel extraction kit (Qiagen, see Table 2.2).

2.2.8 Phenol/Chloroform extractions

Phenol was added to half the volume of the DNA solution and mixed by vortexing for 1 min. The mixture was left at RT for 5 min and then re-vortexed for 1 min. A similar volume of chloroform:isoamyl alcohol (24:1) was added to extract the phenol. The mixture was vortexed for 1 min and then centrifuged for 5 min at 14.000 rpm. The upper aqueous phase was removed and placed in a fresh Eppendorf tube. Ethanol precipitation followed all phenol/chloroform extractions.

2.2.9 Ethanol precipitation

Ethanol precipitation was carried out by adding 3 M sodium acetate to 1/10 the volume of the DNA solution and twice the total volume of 100% ethanol. The sample was placed at -20°C for 30 min and the precipitated DNA collected by centrifugation at 14.000 rpm for 5 min. The supernatant was washed with 100 ml 70% ethanol and air dried for 30 min. The pellet was resuspended in TE buffer (see Table 2.1).

III. ENZYMATIC MANIPULATION OF DNA

2.2.10 Restriction endonuclease DNA digestion

Routine digestion of DNA fragments (0.1-5 μg of DNA) were carried out using 2 U of a restriction endonuclease per μg DNA in the presence of the appropriate buffer supplied by the manufacturer. Reactions were typically incubated for 1-2 h at the recommended temperature in a final volume of 20 μl final volume and terminated by heating to a temperature (65-85°C) sufficient to denature the endonuclease. Digesting DNA samples of more than 5 μg involved scaling the reaction appropriately with respect to amount of restriction endonuclease and the final volume of the reaction.

2.2.11 Dephosphorylation of plasmid DNA

Dephosphorylation of linearized plasmid vector DNA was carried out to prevent religation of plasmid ends. Calf intestinal phosphatase (CIP) was added at a final concentration of 0.5 U/μg plasmid in One-Phor-All buffer (Pharmacia) and incubated at 37°C for 15 min. Another 0.5 U/μg plasmid of CIP was added and incubation at 37°C continued for a further 15 min. Phenol/chloroform extraction (see Sections 2.2.8) followed by ethanol precipitation (see Section 2.2.9) removed contaminating proteins and the DNA resuspended in TE buffer.

2.2.12 Ligation

Ligation of DNA fragments was performed by combining the DNA components at a molar ratio of 10:1 insert:vector in the presence of 1x T4 DNA ligase buffer and 400 U ligase T4 DNA ligase in a final volume of 20 μl. Sticky end ligations were incubated at 22°C for 12 h and half the ligation mix used to transform competent *E. coli* cells (see Section 2.2.2). In all

ligations, a negative control was performed by replacing the DNA insert component with an equal volume of water to establish background ligation due to vector religation.

IV. MUTAGENESIS

2.2.13 U.S.E single strand mutagenesis

Mutagenesis was performed using the U.S.E Mutagenesis Kit supplied by Pharmacia according to the manufacturer to convert the plasmid pPR2hIL5 (see Table 2.5) into pPR2hNKA. Briefly, the mutagenic primer hNLS3NKA (see Table 2.6) designed to remove a Bal I restriction site and insert an Stu I restriction site was annealed to the pPR2hIL5 plasmid and incorporated into a nascent strand by in vitro DNA synthesis, using T4 DNA polymerase. The DNA was then treated with the Bal I restriction enzyme whereby plasmid DNA which incorporated the mutagenic primer was resistant to digestion, while wt plasmid DNA was linearized. The DNA mixture was then introduced into *E. coli* strain NM522 mutS (see Table 2.3), a strain whose defect in repairing mismatches in duplexed DNA helps ensure against repair of the mutations during growth. Since covalently closed circular DNA will be taken up by bacteria 100- to 1000-fold more efficiently than linear DNA, the majority of the transformants would contain mutant plasmids. Plasmid DNA was then isolated from the transformed cells and subjected to a second round of Bal I restriction enzyme selection to increase the proportion of mutant plasmids. The mixture was then transformed into *E. coli* strain MC1060 cells (see Table 2.3) and screened for clones containing the mutation based on restriction analysis with Bal I and Stu I endonucleases.

2.2.14 Double-stranded mutagenesis

Mutagenesis was performed according to the QuikChange Site–Directed Mutagenesis Kit protocol supplied by Stratagene according to the manufacturers instructions. Briefly, the QuikChange site-directed mutagenesis method was used with double-stranded plasmid DNA. The procedure starts with a supercoiled, dsDNA vector with the insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, were extended during temperature cycling by native PfuTurbo DNA polymerase under the following conditions:

Cycle number	Time per cycle	Temperature
1cycle	30 sec	95°C
16 cycles	30 sec	95°C
	1 min	55°C
	2 min per kb of plasmid length	68°C

On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks was generated. After temperature cycling, the product was treated with 10 units of the restriction endonuclease Dpn I for 1 hour at 37°C to digest the parental DNA template and select for the synthesized DNA containing mutations. Since DNA isolated from most *E. coli* strains is *dam* methylated, it is susceptible to Dpn I digestion, which is specific for methylated and hemi-methylated DNA. The reaction was stopped by heating the mix for 10 min at 68°C. The nicked vector DNA incorporating the desired mutations was then transformed into the competent *E.coli* strain Top 10 F' (see Table 2.3). The cells were grown overnight on LBA plates and mutant colonies verified by colony PCR (see Section 2.2.16).

2.2.15 Polymerase chain reaction

PCR was carried out to amplify DNA fragments for cloning and to screen for recombinant plasmids. For the purpose of construct preparation, the high fidelity Pfu DNA polymerase was used while for routine analysis/screening, Taq DNA was utilized according to the protocols described in Table 2.10.

All components were combined on ice and the reactions carried out under the following cycling conditions: pre-amplification denaturation was carried out at 94°C for 30 s followed by 30 thermal cycles consisting of denaturation (94°C for 30 s), primer annealing (for 30 s at temperatures 4-10°C below T_m of the primers) and extension (72°C for 1 min (Taq) or 2 min (Pfu) per kb of the product). 10 µl of the PCR product was analysed by agarose gel electrophoresis (see Section 2.2.5).

Table 2.10. PCR reaction components incorporating Pfu or Taq DNA polymerase.

Pfu reactions	**Taq reactions**
1x cloned Pfu buffer	1x Taq buffer
250 μM each dNTP	250 μM each dNTP
250 nM each primer	250 nM each primer
2.5 units Pfu turbo enzyme	0.5 units Taq enzyme
2 ng/μl plasmid template DNA	2 ng/μl plasmid template
	2 mM $MgCl_2$
H_2O up to 50 μl	H_2O to 20 μl

2.2.16 Colony PCR

For screening of mutant *E. coli* colonies, transformed cells were grown overnight on LBA plates. Single colonies were picked with a sterile toothpick and spotted onto a fresh LBA master plate before resuspending in 50 μl water, heated for 10 min at 65°C and the lysate placed on ice. Each PCR reaction contained 5 μl lysate, 1x Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.25 pmol of each primer and 0.55 units Taq polymerase. PCR was performed as shown in Table 2.11.

Table 2.11. Thermal profile for colony PCR reactions.

Cycle number	**Time per cycle**	**Temperature**
1 cycle	1 min	94°C
30 cycles	30 sec	94°C
	15 sec	T_m-2°C
	1 min per kb of plasmid length	72°C

The DNA samples were amplified using a capillary thermal cycler (FTS-1, Corbett Research, Australia). PCR products were separated for analysis on a 1.5% agarose gel with subsequent staining in ethidium bromide for 30 min.

2.2.17 DNA sequencing

The fidelity of clones was determined by automated sequencing by combining 500 ng of plasmid, 3.2 pmol sequencing primer, 8 µl BigDye Terminator Mix and water in a final volume of 20 µl. The mixture was then subjected to 25 thermal cycles involving 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Unincorporated dye-labelled terminators were separated from the mixture by ethanol precipitation by adding 2.0 µl 3M NaAcetate and 50 µl 100% EtOH. The mixture was incubated on ice for 7 min and then centrifuged at 14.000 rpm for 30 min at RT. The supernatant was discarded and the pellet washed with 500 µl 70% EtOH. The pellet was then dried and the sequence determined using the Perkin Elmer ABI Prism 377 DNA sequencer in the Biomolecular Research Facility, JCSMR, ANU.

2.2.18 Mammalian cell transformation

FDC-P1 cells were grown in RPMI-1640 completed medium with the factor mIL-3 (see Table 2.1) and subcultured at 5 x 10^5 cells/ml the day before transfection. The cells were harvested and resuspended in factor-free growth medium described above. 7.5 x 10^6 cells were incubated with 10 µg of the plasmid pcDEF3mIL5Rα (see Table 2.5) in 350 µl of growth medium supplemented with 20% FCS for 15 minutes at room temperature, and subjected to electroporation at 300 V and 500 µF capacitance (Bio-Rad GenePulser). The cells were transferred into 10 ml of fresh medium and selected for receptor-expressing cell by culturing with the factor mIL-5 over a period of 4 to 8 weeks.

2.3 Analysis of Proteins

I. QUANTITATION OF PROTEINS

2.3.1 Bradford Assay

The concentration of proteins was determined using the Bio-Rad Bradford assay (Bradford, 1976) according to manufacturer's specifications. Solutions containing 2-14 µg of the protein standard BSA were made up to 0.8 ml in water while 3-5 different dilutions of the protein to be assayed were prepared and made up to 0.8 ml. 0.2 ml of the protein binding reagent (Bio-Rad) was added and incubated for 10 min at RT. The absorbance at 595 nm was measured and the values subtracted from a reagent blank prepared from 0.8 ml distilled water and 0.2 ml of the above reagent. The concentration of protein was calculated by extrapolating from the standard curve obtained from BSA of known concentration (0.2 mg/ml).

II. ELECTROPHORETIC SEPARATION OF PROTEINS

2.3.2 SDS-PAGE

Proteins were separated using SDS PAGE. Purity and molecular weight of proteins were verified using 10% acrylamide gels. A 10% acrylamide gel consisting of 10% acrylamide (29:1 acrylamide:bisacrylamide), 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 0.03% AMPS and 0.015% TEMED was poured and allowed to set for 1 h at RT. The stacking gel containing 4.5% acrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 0.03% AMPS and 0.015% TEMED was poured on top of the resolving gel with a comb inserted and allowed to set at RT for 1 h. For immunoblotting analysis of proteins, 4-20% gradient gels were used (Gradipore).

Protein samples were prepared for loading by adding SDS loading buffer (Table 2.1) in a 1:1 ratio, followed by boiling for 5 min, vortexing for 1 min, and centrifugation at 14.000 rpm for 5 min. Electrophoresis was performed in 1x SDS buffer (Table 2.1) at 70 V until the bromophenol blue dye marker reached the bottom of the gel. Gels were either prepared for Western blotting procedure or Coomassie staining (see Section 2.3.3).

III. PROTEIN DETECTION

2.3.3 Coomassie Staining

Gels were stained in Coomassie Blue Solution (Table 2.1) overnight and destained for 2-5 h with successive changes of Destaining Buffer (Table 2.1). The molecular weight was compared to a lane loaded with a standardized prestained molecular weight makers (Benchmark, Gibco). Protein concentration was determined using the Bradford assay (see Section 2.3.1).

IV. PROTEIN LABELLING

2.3.4 Fluorescent labelling of proteins

Labelling of purified β-gal fusion protein was performed by adding 1/10 the volume of 4 mg/ml 5-iodacetamido-fluorescein (IAF, Molecular Probes, Inc. USA, 200 µl) dissolved in 1 M Bicine (pH 7.8). The solution was adjusted to pH 7.8 with 1 M KOH and incubated at RT for 90 min wrapped in aluminium foil. To remove free dye, 2 ml of labelled protein were loaded on to a PD-10 column (Pharmacia) to separate the labelled protein from unincorporated dye. The samples were eluted with 3 ml of PBS, and then concentrated and purified using centricos (Millipore). To calculate the ratio of label to protein, 1/500 or 1/250 dilutions of the purified fusion proteins were prepared in 1 ml of H_2O, and the concentration and extent of labelling of the protein determined based on absorbance readings (A) at 495 nm (label) and 280 nm (protein) according to the following equations (Rihs and Peters, 1989):

$$\text{Concentration (mg/ml)} = \frac{A280 - (0.35 \times A495)}{1.8} \times \text{dilution factor}$$

$$\text{Labelling (mol/mol)} = \frac{A495 \times 1.8}{0.15 \times [A280 - (0.35 \times A495)]} \times 0.233$$

Baculovirus-expressed cytokines and cytokine receptors were fluorescently labelled using the covalent amine-labelling reagent SFX (fluorescein-X succinimidyl ester). 100 µg of freshly prepared SFX (5 mg/ml in DMSO) was added to 200 µg of protein PBS and

incubated at RT for 1.5 h, protected from light. PBS was then added to bring the sample volume to 500 µl, and the total volume was loaded on to a NAP-5 column (Pharmacia) to separate the labelled protein from unincorporated dye. The samples were eluted with 1 ml of PBS, protein samples concentrated using `Ultrafree-MC' filter units (Millipore) and concentration measured using Bradford assay (see section 2.3.1)

2.3.5 Biotinylation of proteins

To biotinylate mIL-3 and mIL-5, a N-hydroxysuccinimide-Biotin (Sulfo-NHS-Biotin) was utilized. Sulfo-NHS-Biotin is a water-soluble, membrane-impermeable biotin derivative, which reacts with primary amines of proteins. It contains a 22.4 Å long, non-cleavable spacer for improved complex formation with streptavidin and reduced steric hindrance.
Biotinylation was performed according to the manufacturers instructions (Pierce). 500 µl of baculovirus-expressed mIL-5 and mIL-3 were loaded on to a NAP-5 column (Pharmacia) and eluted with 1 ml PBS pH 7.5. The protein samples were concentrated using a 10kD MWCO `Ultrafree-MC' filter units (Millipore) and the protein concentration adjusted to 1 mg/ml after determining the concentration using the Bradford assay (see Section 2.3.1).
To 100 µl of 1 mg/ml mIL-5 or mIL-3 were added 40 µl of 4 mg/ml biotin dissolved in PBS pH 7.5 and incubated overnight at 4°C. To remove unincorporated biotin, sample volumes were adjusted to 500 µl with PBS and samples loaded on to NAP-5 columns and eluted with 1ml PBS. The purified protein samples were concentrated using 10 kD MWCO `Ultrafree-MC' filter units, the protein concentration determined by Bradford assay and adjusted to 1 mg/ml.

2.4 Expression and purification of β-gal fusion proteins

2.4.1 Expression

Bacteria containing the plasmids encoding the β-galactosidase fusion proteins used in this study were inoculated into 5 ml LB with 100 µg/ml ampicillin and grown at 37°C overnight (220 rpm). The culture was transferred into 400 ml LB containing 100 µg/ml ampicillin and 1 mM IPTG for fusion protein induction, and grown at 37°C overnight with

shaking (220 rpm). The bacteria were harvested by centrifugation at 8.000 rpm for 15 minutes at 4°C (GS-3 rotor) and resuspended in 5 ml of Protein Incubation Buffer (see Table 2.1).

2.4.2 Purification

Cells were disrupted at 4°C by five 3 minute cycles of ultrasonication at 3-4 output with a 1 minute break between cycles using a Model W-220F sonicator (Ultrasonics Inc. USA) and then transferred into 1.5 ml Eppendorf tubes. After centrifugation (SS34 rotor) for 30 minutes at 4°C at 13.000 rpm, the supernatant was collected and NaCl added to a final concentration of 1.6 M. The supernatant was then applied to a prewashed Econo-column (Bio-Rad) containing 4 ml of p-aminobenzyl-1-thio-β-D-galactopyranoside-agarose (Sigma), which contains a covalently attached nonhydrolyzable β-galactosidase substrate. The column was then extensively washed with 2 litre of Equilibration Buffer (see Table 2.1) to remove non-specifically bound material. The fusion protein was eluted using Elution Buffer (see Table 2.1). 15-20 fractions were collected into Eppendorf tubes containing 250 µl of 2 M Tris-HCl (pH 7.0) for immediate neutralization of the eluate. Tubes were mixed by inversion and stored on ice.

β-galactosidase activity in fractions was checked by adding 20 µl of orthonitrophenyl-β-D-galactopyranoside (ONPG, Sigma, 4 mg/ml) to 10 µl of each fraction. Fractions exhibiting a yellow colour change were subsequently analysed by SDS-PAGE (see section 2.3.2). The gel was stained in Coomassie Blue Solution (Table 2.1) for 1 hour and destained for 2-3 hours by repeatedly changing the Destaining Buffer (Table 2.1). Column fractions were stored at -20°C.

Based on the results of the gel analysis, peak protein fractions were dialyzed in a 50 kDa MWCO dialysis membrane against 2 litres of Dialysis Buffer (Table 2.1) at 4°C with continuous stirring overnight. Dialysed fusion proteins were transferred into a 100/75 (75 kDa MWCO) ultrathimble tube (Schleicher and Schüll, Germany) and pressure dialyzed against 200 ml of Dialysis Buffer (see above) using a vacuum pump (Edwards, England) to concentrate the protein solution. The protein concentration was determined by measuring the absorbance at 280 nm with 1.8 optical density (OD) for 1 mg/ml of protein (Rihs and Peters, 1989).

2.4.3 Filter-based protein concentration (Centricon, Millipore)

E.coli expressed β-gal fusion proteins were concentrated and purified from label at 3,500 rpm (SS34 rotor) using a 100 kDa MWCO centricon (Amicon, USA). Repeated cycles were carried out of washing the centricon with Injection Buffer (Table 2.1). When the flowthrough was free of chromophore as indicated by examination under UV (UV transilluminator, USA), the labelled protein was collected, its absorbance measured at 280 and 495 nm and then stored at -70^{o}C until further use.

2.5 Expression and purification of recombinant cytokines and cytokine receptors

hIL-5, mIL-5 the NLS1 and the monomeric mutant derivative of mIL-5 were provided by the Cytokine Molecular Biology and Signalling Group, JCSMR. hIL-5, mIL-5 and the NLS mutant derivatives of mIL-5 were expressed using the baculovirus system in Sf9 insect cells. The proteins were purified by gel filtration followed by ion-exchange chromatography and activity measured using a Thymidine incorporation assay as described in Section 2.7.3.

2.6 Expression and purification of IL-5_{sc}-HSA fusion protein

The IL-5_{sc}-HSA fusion protein was provided by the Cytokine Molecular Biology and Signalling Group, JCSMR.

The plasmid expressing the hIL-5_{sc}-HSA fusion protein was derived by PCR of 3 separate fragments with subsequent subcloning into the baculovirus expression vector pBakPak8. First, a hIL-5 fragment, including the signal sequence and flanked by Bam H I/Nar I restriction sites was derived by PCR and inserted into the MCS of the plasmid pBSKS^{+}. A second IL-5 fragment, without the signal sequence and flanked by Nar I/Kpn I sites was derived and subcloned in identical fashion. This single-chained IL-5 construct was then subcloned into the MCS of the transfer vector pBakPak8. Finally, a PCR product consisting of HSA sequence encoding for aa 25-588 (CAA23754.1) flanked by Kpn I/EcoR I sites was

inserted into pBakPAC8. Constructs were screened by restriction analysis and a positive clone sequenced to confirm its identity.

The hIL-5_{sc}-HSA fusion protein was baculovirus-expressed and purified by the Cytokine Molecular Biology and Signalling Group, JCSMR as described in Section 2.6 and fluorescently labelled as described in section 2.3.4 of Material and Methods.

2.7 Cell culture

2.7.1 HTC cells

Cells of the rat hepatoma tissue culture (HTC) line, a derivative of Morris hepatoma 7288C cell line, (Flow Laboratories, Bonn, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM, ICN Biomedicals, Inc. USA, (Jans et al., 1991; Rihs et al., 1991) supplemented with 10% heat-inactivated foetal calf serum (FCS, CSL) in a humidified incubator (Forma Scientific, Inc. USA) with 5% CO_2 atmosphere at 37°C. The cells, grown in 50 ml flasks (Nunclon, Denmark), were maintained by Lyndall Briggs, Anna John and Chenoa Barton (Nuclear Signalling Laboratory, JCSMR). Cells were subcultured every 3-4 days (at 90-100% confluence) in a laminar flow hood (Airpure, Email Westinghouse Pty. Ltd. Australia) by removing the medium, washing the cells twice with sterile PBS and resuspending them by incubating with 2 ml of 0.25% trypsin in EDTA (Sigma) diluted 1 in 10 in PBS for 3-5 min at 22°C. The trypsinised cell suspension was transferred into a 50 ml Falcon tube containing 6 ml fresh DMEM containing 10% FCS and centrifuged at 500 rpm (Sorvall Technospin) for 5 min at 22°C. After discarding the supernatant, the pellet was resuspended in 5 ml DMEM. For maintenance, 0.2 and 0.5 ml of cells were pipetted into fresh culture flasks.

2.7.2 FDC cells

The mouse myeloid progenitor FDC-P1 factor (IL-3)-dependent cell line was provided by Sally Ford (Cytokine Molecular Biology and Signalling Group, JCSMR) for further culturing as well as for transformation with the pcDEF3mIL5Rα (see Table 2.5) plasmid to obtain the FDC-mIL-5R cell line (see section 2.2.18). These cells were grown in RPMI-1640 medium as listed in Table 2.1.

The mIL-3 and mIL-5 used for culturing was prepared from Sf9 cells infected with a baculovirus expressing mouse IL-3 and mouse IL-5 and was provided by S. Ford (JCSMR). One unit of factor bioactivity is the amount of IL-2 required to support half-maximal proliferation of the corresponding FDC-cells. Every three days cells were diluted 1 in 10 and the medium was replaced with fresh medium containing the appropriate factor. The cells were grown at 37°C, 5% CO_2 in air and 95% relative humidity.

2.7.3 Bioassay (^{3}H-Thymidine incorporation)

For routine determination of bioactivities of baculovirus-expressed factors, a ^{3}H-thymidine incorporation assay was performed by Sally Ford, Cytokine Molecular Biology and Signalling Group, JCSMR.

FDC cells, expressing the appropriate receptor, were grown to $4x10^5$ cells/ml and washed 4 times in factor free RPMI-1640 medium to remove any factors and resuspended in RPMI to $2x10^4$ per 50 µl.

15 serial dilutions of factor were pipetted in triplicates into 96 well plates, with a starting concentration of 300 nM and then 50 µl of cells added. One well was left as blank and cells added to factor-free media.

Cells were incubated for 44 hours at 37°C, 5% CO_2 in air and 95% relative humidity. Subsequently, cells were pulsed with 0.5 µCi of ^{3}H-thymidine per well, cells harvested after 4 hours using a cell harvester (Inotech) and ^{3}H-thymidine incorporation counted using a liquid scintillation counterr (Packard, Australia). Data analysis was performed using the Kaleida Graph 2.13 software (Macintosh).

2.7.4 Biotinylation of cell surface proteins

FDC cells were washed 3 times with ice-cold PBS (pH 7.5) to remove any media and to adjust the pH to slightly alkaline. Cells were resuspended in PBS to a concentration of $2x10^6$ cells/ ml and 0.5 mg of Sulfo-NHS-LC-Biotin per ml added. Cells were incubated for 30 min at RT and then washed 3 times in ice-cold PBS to remove any remaining biotin derivative (Altin, 1995).

2.8 Confocal laser scanning microscopy (CLSM)

The localisation of fluorescently tagged proteins within the cell was monitored by CLSM. This technique is based on fluorescence microscopy whereby a light source is passed through two sets of filters - one to ensure that light reaching the tissue specimen is of the optimal wavelength to excite the designed fluorescent chromophore, and one to filter the light obtained from the specimen to allow only wavelengths specifically emitted when the chromophore fluoresces to be collected. CLSM is similar in this regard except a laser is used to illuminate the specimen through a pinhole aperture at a single point, and only emitted-light from the focal plane is allowed to pass through the same pinhole to reach the detector. By scanning the beam of light across the specimen, very sharp two-dimensional images of the exact plane of focus can be built up. Rapid scanning (1 scan per second) enables experiments to be carried out in real time on living cells. Due to its special optics and higher sensitivity digitized image, the CLSM can be used to measure relative amounts of fluorescently labelled proteins at the subcellar level in living cells.

2.9 *In vitro* nuclear transport assay

For nuclear transport assays, 0.8 and 1.0 ml of trypsinised cells (Section 2.7.1) were pipetted into two fresh tissue culture flasks containing 24 ml fresh DMEM. HTC cells were seeded in DMEM on coverslips (15 mm x 15 mm) in 12 well culture plates (Nunclon, Denmark) and grown for 36 h to 50-70% confluence. The DMEM was then replaced with 2 ml of DMEM without phenol red and glutamine (ICN) containing 1 M Hepes with 10% FCS prior to use for analysis of nuclear import. Nuclear import was analysed at the single cell level using mechanically perforated HTC cells in conjunction with CLSM (see Section 2.8) (Efthymiadis et al., 1998; Jans et al., 1991; Xiao et al., 1996). Briefly, HTC cells grown on glass coverslips were rinsed with 1x IB (see Table 2.1). Excess liquid was drained followed by adding 3 µl 1x IB. A single layer of tissue paper was placed onto the cell monolayer and rapidly removed 3 seconds later to mechanically perforate the cells. The coverslip with perforated cells was then placed with the cell-bearing surface face down to the nuclear import mix, which had been prepipetted into a chamber on a slide created by 0.1-mm-thick double-sided pressure-sensitive tape (Scotch 3M) into which a hole of 0.8

mm in diameter was punched.

Experiments were performed in a 5 µl volume containing BSA (30 mg/ml) or cytosolic extract (untreated reticulocyte lysate, Promega), an ATP-regenerating system (0.125 mg/ml creatine kinase, 30 mM creatine phosphate, 2 mM ATP) and transport substrate or a control (70-kDa fluorescein isothiocyanate- or Texas-red-labelled dextran; Sigma) to assess nuclear integrity. In inhibition experiments, cytosolic extract or the transport substrate was preincubated at RT for 15 min with antibody (40 µg/ml) specific to Importin β (Santa Cruz). The coverslip was finally sealed with nail polish to prevent dehydration during the experiment at RT.

Scanning over time (every 3-4 min over 15-45 min) was performed using a Nikon Optiphot or Bio-Rad 2000 microscope, using a 60x magnification oil immersion lens.

Image analysis and curve fitting was performed as described (Efthymiadis et al., 1997; Xiao et al., 1997). Images obtained from CLSM were analysed using the NIH Image 1.62 public domain software. The ratio (Fn/c) of nuclear (Fn) to cytoplasmic fluorescence (Fc) was determined as follows:

$$Fn/c = (Fn - Fb) / (Fc - Fb),$$

where Fn/c was the nuclear/cytoplasmic ratio, Fn the fluorescence in the nucleus, Fc the fluorescence in the cytoplasm, and Fb the background fluorescence due to autofluorescence. Experimental data for nuclear import kinetics over time was fitted with the following equation :

$$F_{n/c}(t) = Fn/c_{max}(1 - e^{-kt}),$$

using the KaleidaGraph 2.13 software (Macintosh). Fn/c (t) represents the nuclear/ cytoplasmic ratio at time t, Fn/c_{max}, the maximal level of nuclear accumulation, and k the import rate constant.

2.10 ELISA

The binding of bacterially expressed GST-tagged mouse importins to IL-5NLS β-gal fusion proteins and IL-5_{sc}-HSA fusion protein was assessed using an ELISA-based binding assay (Efthymiadis et al., 1998; Efthymiadis et al., 1997; Hübner et al., 1997; Xiao et al., 1997). Proteins were coated into the wells of polystyrene microtiter plates (Nunclon) in triplicates at 0.5 μg/well using 50 mM NaHCO3 (pH 9.8) for 16 h at 4°C. The wells were blocked by adding 400 μl 1x IB containing 1% BSA for 1 h at RT with shaking (Platform rocker; Bio-Line), and then washed 4 times with 1x IB (Table 2.1) with the third wash being incubated for 1 h at RT as above. Serial dilutions of importins were then added in Hybridisation buffer (Table 2.1) to the microtiter plates and incubated for 16 h at 4°C. After extensive washing with 1x IB containing 1% BSA, goat GST-specific antibody (500 ng/well) was added and the plates incubated for 3 h, and non-specifically bound antibody removed by washing 10 times with PBS containing 0.3% Tween-20 (Bio-Rad). Alkaline phosphatase-conjugated rabbit anti-goat IgG (0.025 U of enzyme/well) was added and the plates incubated for 1 h at RT and washing performed as described for the primary antibody. Binding activity was then determined by adding the chromogenic substrate para-nitrophenyl phosphate (p-NPP, 1 mg/ml, Sigma) dissolved in 10% diethanolamine and 0.5 mM $MgCl_2$ (pH 9.8) and the change of absorbance at 405 nm followed over 90 min using a plate reader (Molecular Devices, Menlo Park, Ca, USA), with values corrected by subtracting both the absorbance at 0 min, and that in wells incubated without importins. Data were collected and stored using the Softmax software program (Macintosh). Processing of the ELISA raw data and correction of values were carried out using the Microsoft Excel software (Efthymiadis et al., 1997; Hübner et al., 1997). Binding affinity of proteins or peptides to importin subunits were evaluated by plotting the corrected absorbance for different importin concentrations against time using the KaleidaGraph 2.13 software (Macintosh). Data in the linear range were used to obtain the OD change per min (OD/min), which was plotted against the concentration of importins. Curves obtained were fitted using the function: $B(x) = B\ max\ (1- e^{-kx})$, where x is the concentration of importin, and B is the level of importin bound. The Kd, the apparent dissociation constant, representing the concentration of importins yielding half-maximal binding, was calculated from this equation.

2.11 Post-Embedding Immunogold Staining (Electron microscopy)

Samples for electron microscopy were prepared following a procedure adapted from (Dvorak, 1989). Cells in suspension were washed 3 times in PBS and subsequently fixed in fixation buffer, containing 4 % PFA and 0.25 % glutaraldehyde (see Table 2.1). After fixation for 2 hours at 4°C, cells were washed in S-PBS (see Table 2.1) and then incubated for 1 h in SN-PBS containing 50 mM NH_4Cl (see Table 2.1). Thin sections were prepared by the Electron microscopy unit, JCSMR.

For immunostaining, thin sections were first incubated in blocking solution (see Table 2.1) for 30 min followed by incubation with the primary antibody for 18 h at 4°C. Cells were washed 3 times in PBS before incubation with the gold-labelled secondary antibody at 4°C for 1 h. Cells were washed 3 times in PBS, then contrasted with uranyl acetate and lead citrate and subsequently washed 6 x 1min in H_2O. Samples were viewed and photographed using a Philips EM301 electron microscope at 100 KV.

2.12 Flow cytometry

2.12.1 Flow cytometric studies of ligand-receptor interactions

FDC-P1 cells grown to $1x10^6$ cells/ml, washed 3 times with factor-free RPMI (0.1%BSA), and resuspended in cold PBS and aliquoted to 10^5 cells per sample. The cells were incubated with IL-5~Sfx for total binding or IL-5~Sfx with IL-5 in 100-fold excess for 30 min on ice to avoid internalisation of the ligand.

Flow cytometric measurements were performed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Inc.) with instrumental setting at FL1 for measurements of fluorescein fluorescence (laser excitation at 488 nm and emission at 530 nm, bandwidth 30 nm). Live cells were gated by light scattering or by exclusion of propidium iodide, and 10 000 events were acquired in each scan.

The geometrical mean of the fluorescence intensity of gated cells was determined using CellQuest software (Becton Dickinson) for Macintosh. Subsequent analysis of data was performed using the Kaleida Graph 2.13 software (Macintosh).

2.12.2 Cell viability

Prior to subcellular fractionation, cells were tested for viability using flow cytometry to ensure that the percentage of live cells was greater than 95%. $5x10^5$ cells were resuspended in 500 µl PBS and 10 µl of propidium iodide (150 µg/ml) added and incubated for 10 min on ice. The fluorescence of cell nuclei as a measure of cell death was determined using the FACScan flow cytometer with instrumental settings at FL2 for measurements of propidium iodide fluorescence (laser excitation at 488 nm and emission at 575 nm, bandwidth 42 nm). Data were analysed using the CellQuest software.

2.13 *In vivo* fluorescence studies

For *in vivo* subcellular localization studies, 10^7 FDC-mIL-5R cells were subcultured in factor free RPMI-1640 medium in 1 µM fluorescently labelled mIL-5-Sfx in the absence or presence of a 10-fold excess of unlabelled mIL-5 for various times at 37°C. After different times 10^6 cells were harvested into 15 ml centrifuge tubes, fresh factor free RPMI-1640 medium added to make a final volume of 15 ml, and the cells centrifuged at 1500 rpm for 5 min at 18°C. Following removal of the supernatant, the cells were resuspended in 15 ml of medium and the wash repeated three times to remove any remaining factor.

Cells were resuspended in 5 µl of medium and pipetted into a chamber on a slide created by 0.2-mm-thick double-sided pressure-sensitive tape (Scotch 3M) into which a hole of 0.8 mm in diameter was punched. A coverslip was mounted onto the slide and the cells sealed with nail polish to prevent dehydration during the experiment at RT. Cells were kept on ice until visualisation of fluorescence using CLSM.

Image analysis of the CLSM files was performed using the NIH Image 1.49 public domain software to quantitate the membrane and nuclear fluorescence in the absence (total binding) and presence (non-specific binding) of unlabelled hIL-5, with the latter subtracted from the former to give a measure of the fluorescence (specific binding).

2.14 Immunoblotting

2.14.1 Western blotting

Protein samples were transferred from the protein polyacrylamide gels onto nitrocellulose membrane (Schleicher & Schuell) using the Mini Trans-Blot Transfer cell (BioRad).

First, two Whatman paper (Advantec MFS, Japan) and one western blot nitrocellulose transfer membrane (Schleicher & Schuell, Germany) were cut to size (9 x 7 cm & 8 x 6 cm accordingly) per gel. Membranes and gels were equilibrated in transfer buffer (Table 2.1) for 15-30 min, the transfer apparatus assembled according to the manufacturer's specifications and then run overnight at 10-30 V constant voltage.

Transfer efficiency was checked by Coomassie staining the gels, while the membranes were incubated in blocking buffer (Table 2.1) for 60 min at RT on an orbital shaker (Ewards, Australia). The membranes were incubated in primary antibody for 60 min at RT, washed 3x10 min at RT with PBS-T, incubated for 1 h at RT in a secondary HRP-conjugated antibody and washed again 3x10 min at RT with PBS-T. For protein detection the ECL Plus™ Western blotting system (Amersham Pharmacia Biotech) was used according to the manufacturer's specifications. Digital images were derived using the LAS-1000 imaging system (Fuji, Japan).

2.14.2 Stripping and re-probing

Antibodies were removed from immunoblots using Re-Blot plus Stripping solution (Chemicon) according to the manufacturer's specifications. Wet blots were incubated in 1x stripping solution with gentle mixing for 15 min at room temperature, washed in T-PBS and re-probed as described in Section 2.14.1.

2.14.3 Semiquantitative analysis of immunoblots

Semiquantitative analysis of immunoblots was carried out using the Image Gauge software (Fuji) in conjunction with KaleidaGraph 2.13 software (Macintosh).

First, digital images of immunoblots were derived using the LAS-1000 imaging system (Fuji, Japan). The average signal of specific bands as well as background corresponding to each band on the immunoblot was determined and then signal values corrected by subtracting background values of protein-free membrane areas.

2.14.4 Dot Blot

To assess the binding of IL-5 to importins under native conditions, dot blots were prepared with baculovirus-expressed IL-5 or *E. coli* expressed IL-5NLS-β-Gal fusion proteins. 8.4 pmol of IL-5 or 16.8 mol of IL-5 NLS-β-Gal fusion proteins per sample were blotted onto nitrocellulose, the blots washed 3 times with PBS/Tween (0.3 %) and blocked overnight in 1x IB/5 % BSA buffer. Blots were washed 4 times 10 min in 1x IB/1 % BSA buffer and then incubated in pre-assembled Importin-GST mix (Table 2.1) overnight at 4°C. After washing 4 times 10 min in PBS/Tween (0.3 %), blots were incubated in primary anti-GST antibody (1x IB/1 % BSA buffer) for 2 hours at RT, then washed 3 times and incubated with secondary AP-conjugated anti goat IgG antibody (1x IB/1 % BSA buffer) for 60 min at RT. Following 3 washes colouring solution was added and the reaction stopped after 2-15 min by washing the blots with water.

2.15 Cell fractionation and Whole cell lysis

2.15.1 Whole cell lysis

10^7 cells grown in suspension were washed twice in cold PBS and the pellet resuspended in 800 μl RIPA buffer containing Protease inhibitors (see Table 2.1). The cell lysate was freeze-thawed twice in liquid nitrogen and subsequently passed through a 23-gauge needle five times to sheer the DNA in the lysate. The lysate was then centrifuged at 13.000g for 15 mins at 4°C (Eppendorf benchtop centrifuge) and the supernatant transferred into a fresh eppendorf tube and stored at -20°C until further use.

2.15.2 Subcellular fractionation

2×10^7 to 10^8cells in suspension were harvested, washed twice with cold PBS, centrifuged at 1500 rpm for 5 mins at 4°C and then carefully resuspended in 1 ml of NP-40 lysis buffer (see Table 2.1) per 2×10^7 cells. Prior to lysis, cell viability was checked as described in Section 2.12.2. After incubation in lysis buffer for 5 mins, the cells were pelleted by centrifugation at 3000 rpm at 4°C. The pellet containing the nuclei was carefully resuspended and washed twice in 40 ml lysis buffer lacking the detergent NP-40 and then

centrifuged at 3000 rpm at 4°C. The nuclear pellet was resuspended in 150 μl Nuclei lysis buffer (see Table 2.1) per 2 x 10^7 cells, centrifuged at 13.000 g for 5 mins at 4°C, the supernatant transferred into a fresh tube and stored at -20°C until further use.

For fractionation experiments following incubation of cells with biotinylated ligands, 2 x 10^7 to 10^8cells in suspension were first starved for 4 hours in factor-free RPMI-1640 medium (see Table 2.1), the cell viability monitored as described in Section 12.12.2 and then the cells were incubated with the appropriate factor prior to fractionation as described above.

2.15.3 Protein precipitation (Streptavidin-conjugated magnetobeads)

For protein precipitation, subcellular fractions were incubated with 15 µl of magnetobeads (Dynal) per ml lysate. Prior to incubation, beads were washed 3 times with lysis buffer A^+. If cells were incubated with biotinylated factors before lysis, cell lysates were mixed with beads and incubated overnight with constant rotation at 4°C. Beads were pulled down using a magnetic block holding 5 eppendorf tubes (Dynal), washed gently with PBS, and resuspended in 20 µl 2x SDS cracking buffer (Table 2.1). Samples were heated to 120°C for 10 min and loaded onto 4-20% gradient gels (Gradipore).

Electrophoresis was performed in 1x SDS buffer (Table 2.1) at 70 V until the bromophenol blue dye marker reached the bottom of the gel. Gels were prepared for Western blotting and proteins transferred as described in Section 2.14.1.

2.16 Protein-Protein Electrophoretic Mobility Shift Assay (EMSA)

Protein-protein interactions between cytokines and importins were assessed by gel mobility shift analysis. 300 nM of IL-5~biotin was incubated with 3000 nM importins in the presence of gel-shift binding buffer (see Table 2.1) for 20 min at RT. The samples were then subjected to electrophoretic separation through a non-denaturing TBE polyacrylamide gel (4-20%, Gradipore) at 60 V overnight at 4°C. The proteins were subsequently blotted onto Nitrocellulose membrane overnight at 30V, stained with Streptavidin-HRP (Chemicon, 1:10.000), developed with ECL Plus (Amersham), and analyzed on the Fuji Film LAS 1000 using the Image Gauge software.

CHAPTER 3 PATHWAY OF NUCLEAR IMPORT OF IL-5

3.1 Introduction

IL-5 is a cytokine that primarily functions as a stimulus for eosinophil differentiation and activation. Although several IL-5 dependent signalling pathways have been identified, these pathways are shared with IL-3 and GM-CSF and are therefore unlikely to account for the specific role of IL-5 in eosinophil differentiation. A putative bipartite NLS, characterised by two clusters of basic amino acids separated by a spacer of 10-12 amino acids, has been identified in both human and mouse IL-5 (Jans *et al.,* 1997a; see Section 1.4, Fig. 1.8), raising the possibility that targeting of the IL-5/IL-5 receptor complex to the nucleus may play a more direct role in the response of eosinophils to the IL-5 signal.

Mutagenic studies of bipartite NLS such as those of nucleoplasmin (Dingwall *et al.,* 1987), SWI5 (Moll *et al.,* 1991), N1N2 (Hu and Jans, 1999), and retinoblastoma (Rb) protein (Efthymiadis *et al.,* 1997) have established the necessity of both clusters of basic amino acids for nuclear targeting (Li and Etkin 1993; Robbins *et al.,* 1991; Schmidt-Zachmann and Nigg, 1993).

In *in vitro* experiments the hIL-5 bipartite NLS region was able to target the heterologous *E.coli* protein β-galactosidase (476 kDa) to the nucleus. Substitution of lysines 95 and 96 in the proximal arm of the putative NLS region (NLS1) by threonine residues abolished nuclear transport of the fusion protein, suggesting that this bipartite NLS is indeed responsible for targeting IL-5 to the nucleus, presumably not just *in vitro* but also in intact, receptor expressing cells (Jans *et al.,* 1997a).

Additionally, it has been shown for IL-5 that it is capable of co-translocating its receptor subunits into the nucleus *in vitro* (Jans *et al.,* 1997b), with mutation of specific basic residues in the proximal arm (NLS1) abolishing the ability to target its receptor subunits to the nucleus (Calanni, 1997). In contrast, mutations in the distal arm (NLS2) had no impact on the piggyback capacity of IL-5 (Calanni, 1997).

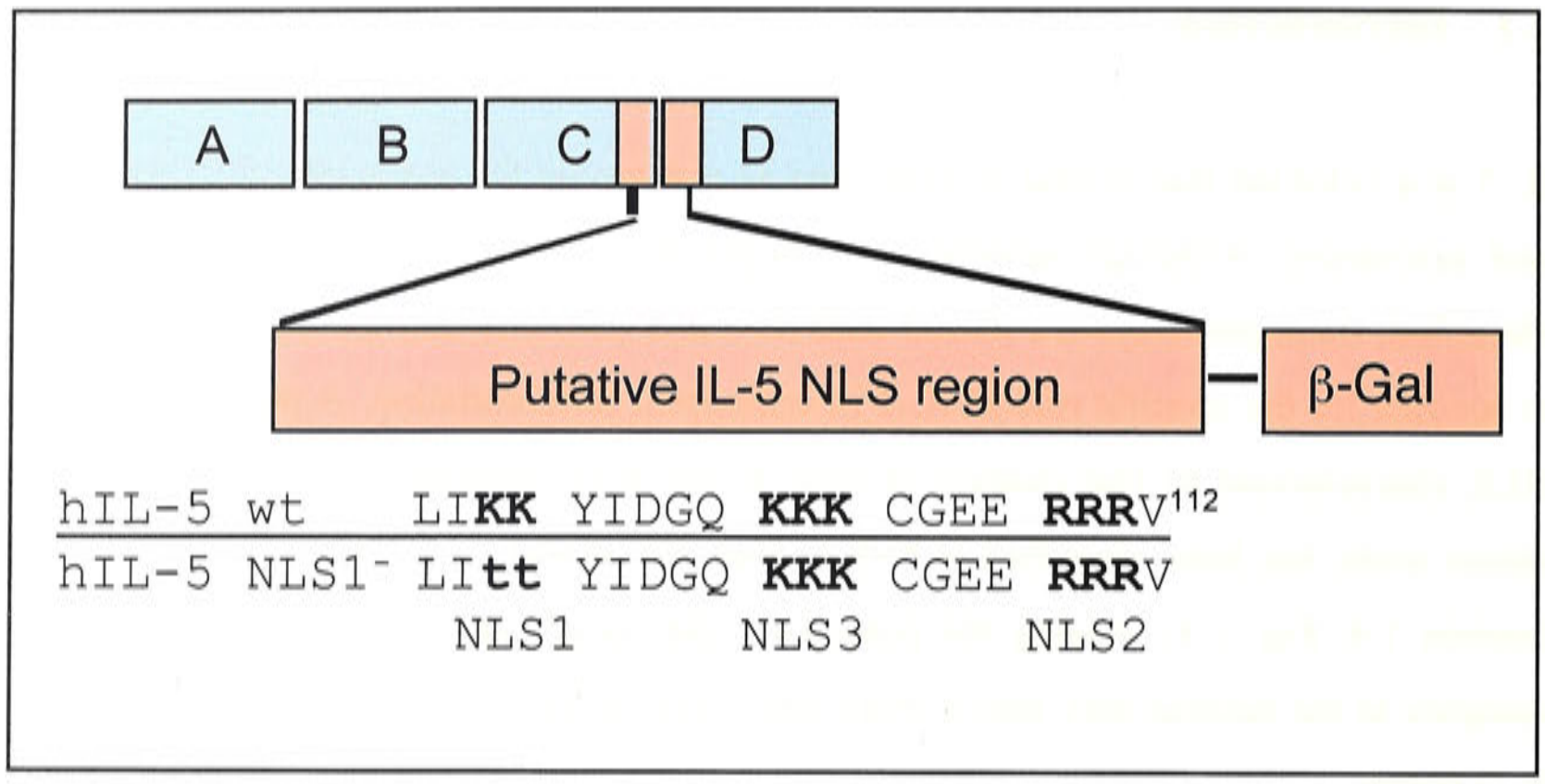


Fig. 3.1. The putative NLS-region of hIL-5. The sequences for hIL-5(wt), and the hIL-5(NLS1$^-$) mutant form are indicated in the single letter amino acid code. Basic residues are indicated in bold.

The aim of the investigations described in this chapter was to characterize the mechanisms by which IL-5 gains access to the nucleus and to investigate the role of putative NLS regions of amino acid residues within the primary sequence of IL-5 in the interaction with components of the cellular nuclear import machinery.

3.2 Flow cytometric approach for measuring the receptor binding capability of IL-5 and its NLS mutant derivatives

Previous experiments have shown that, in contrast to the wild type form, the NLS1 mutant of mIL-5 shows greatly reduced ability to co-transport its IL-5 receptor subunits into the nucleus in an *in vitro* nuclear transport system (Calanni, 1997). In addition, using an *in vitro* eosinophil differentiation assay (unpublished data, Takamoto, Shinshu University School of Medicine, Matsumoto, Japan), it has been shown that while mIL-5 stimulated the production of eosinophil colonies in bone marrow cultures, the mIL-5 NLS1$^-$ mutant derivative was unable to do so. To exclude the possibility that the described phenomena were due to interference of the mutations in the NLS1$^-$ region with high affinity receptor binding, the receptor binding properties of this mIL-5 NLS mutant derivative were examined using a flow cytometry based binding assay.

To establish the feasibility of a flow cytometric approach for measuring the receptor-binding of IL-5~Sfx, the fluorescence flow cytometric profiles of a FDC-mIL-5R cell line were compared with those of the original IL-5 receptor lacking cell lines FDC-P1. The FDC-mIL-5R cells were responsive to IL-5 since they were stably transfected and expressed the mIL-5 receptor α chain in addition to the endogenous mIL-5 β-receptor subunit as described in Section 2.2.18 of Materials and Methods. Both cell lines were exposed to fluorescently labelled mIL-5 and mIL-5 NLS mutant derivatives in the range of 0.2-10 nM. At these low ligand concentrations, cytometric analysis discriminates between ligands associated with the cells and ligands remaining in solution, making removal of unbound ligand unnecessary (Sklar *et al.*, 1984).

The fluorescence cytometric profiles of FDC-mIL-5R cells incubated with mIL-5(wt)~Sfx are shown in Fig 3.2. Panel A shows the total binding of mIL-5(wt)~Sfx to FDC cells expressing the mIL-5 receptor. In a typical experiment, the geometrical mean of the fluorescence of non-treated cells was approximately 3, which arises from cellular autofluorescence. At concentrations of 10 nM the geometrical mean of the fluorescence typically reached a value 2-times greater than that of cells alone.

When the cells were exposed to mIL-5(wt)~Sfx in the presence of a 100-fold excess of unlabelled mIL-5 (Fig. 3.2, Panel B), the fluorescence was drastically reduced, with the residual fluorescence attributable to non-specific binding.

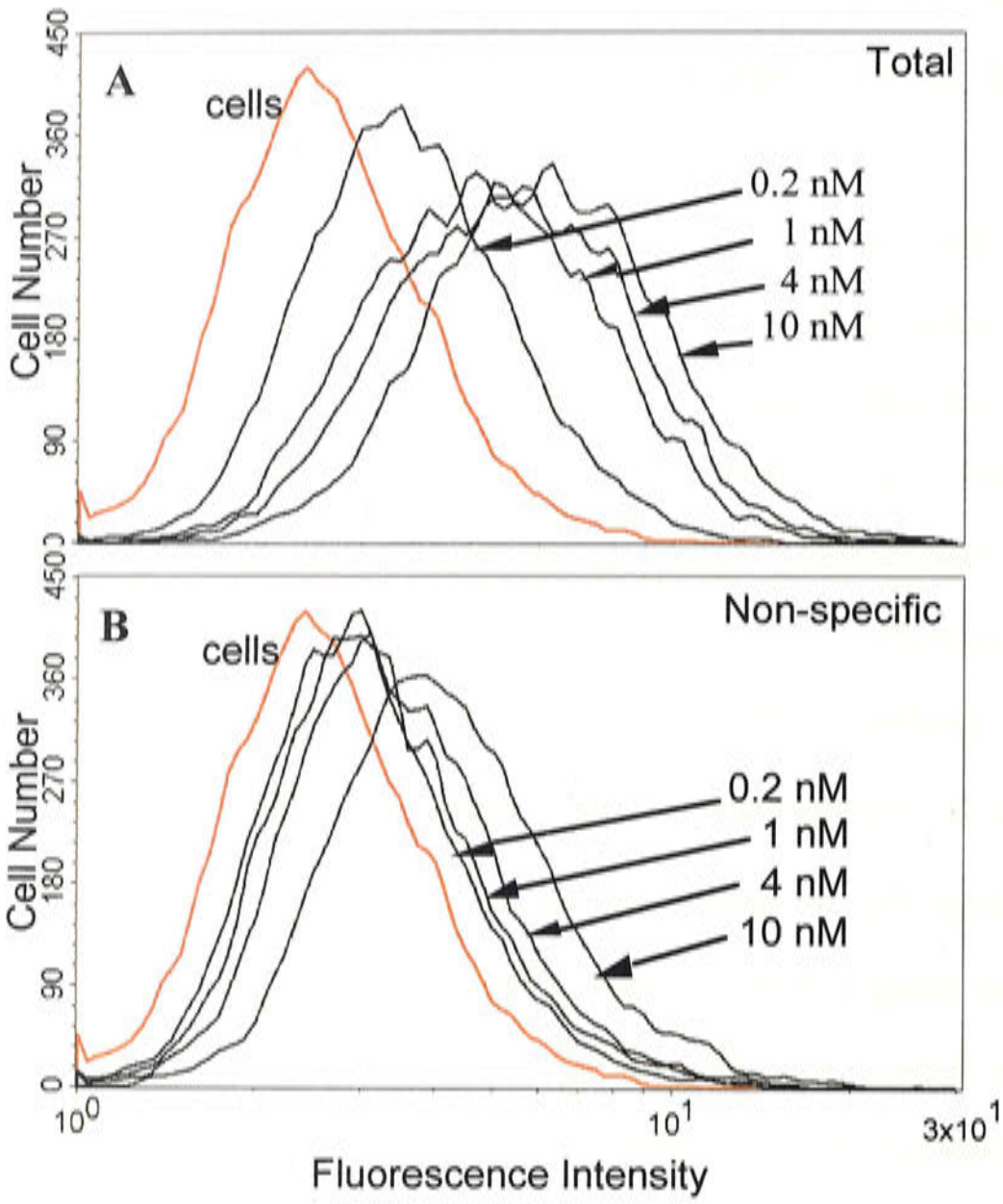


Fig. 3.2. Fluorescence histogram of FDC-mIL-5R cells, incubated with increasing concentrations of mIL-5~Sfx.

Cells of the mouse myeloid progenitor cell line FDC-P1 stably transfected with the cDNA corresponding to the alpha chain of the mIL-5 receptor were incubated for 30 min at 4°C with increasing concentrations of (A) mIL-5~Sfx (total binding) or (B) mIL-5~Sfx in the presence of unlabelled mIL-5 in 100-fold excess (non-specific binding) as described in Section 2.12.1 of Materials and Methods. For clarity, only four sample concentrations of mIL-5~Sfx are shown.

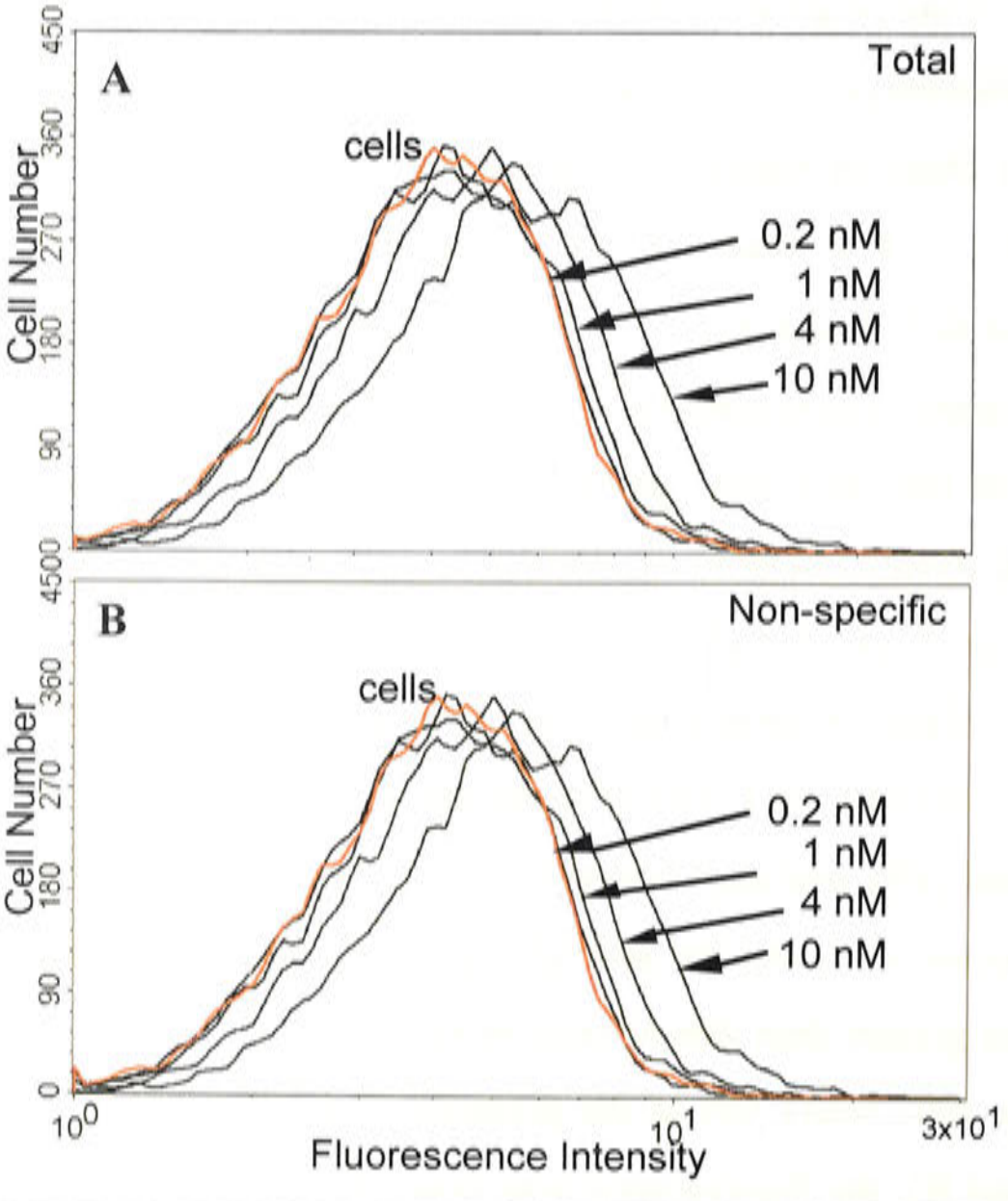


Fig. 3.3. Fluorescence histogram of FDC-P1 cells, incubated with increasing concentrations of mIL-5~Sfx.

Cells of the mouse myeloid progenitor cell line FDC-P1 lacking the mIL-5 receptor alpha subunit were incubated for 30 min at 4°C with increasing concentrations of (A) mIL-5~Sfx (total binding) or (B) mIL-5~Sfx in the presence of unlabelled mIL-5 in 100-fold excess (non-specific binding) at 4°C as described in Section 2.12.1 of Materials and Methods.

The fluorescence cytometric profiles of IL-5 receptor lacking FDC-P1 cells incubated with mIL-5(wt)~Sfx are shown in Fig. 3.3, Panels A and B. The fluorescence in panel A increased only slightly with increasing concentrations of mIL-5(wt)~Sfx, again representing unspecific binding.

Receptor binding curves from these cytometric data were derived by plotting the fluorescence versus the concentration of mIL-5~Sfx. Fig. 3.4, Panel A shows the total and nonspecific binding of mIL-5~Sfx. The fluorescence due to non-specific binding was the fluorescence observed in the presence of unlabelled mIL-5 in 100-fold excess relative to mIL-5~Sfx.

The specific binding of mIL-5~Sfx to FDC-mIL5R cells is shown in Fig 3.4, Panel B. The specific binding is the difference between the total and non-specific binding of mIL-5~Sfx. The data for the specific binding were fitted for the function $B(x) = B_{max}(1 - e^{-kx})$, where B_{max} is the maximal binding, k is the rate constant, and x is the concentration of fluorescently labelled ligand in nM. The k_D value obtained from the binding curve of mIL-5(wt) to FDC-mIL-5R cells was 0.47 nM, which is of the same order of magnitude as published values (150 pM Mita *et al.*, 1989; Koike and Takatsu 1994). In comparison, mIL-5 did not show any significant specific binding to FDC-P1 cells (Fig. 3.4, Panels C and D). These experiments established that a flow cytometric approach could be used to investigate the receptor binding capabilities of the mIL-5 $NLS1^-$ mutant derivative.

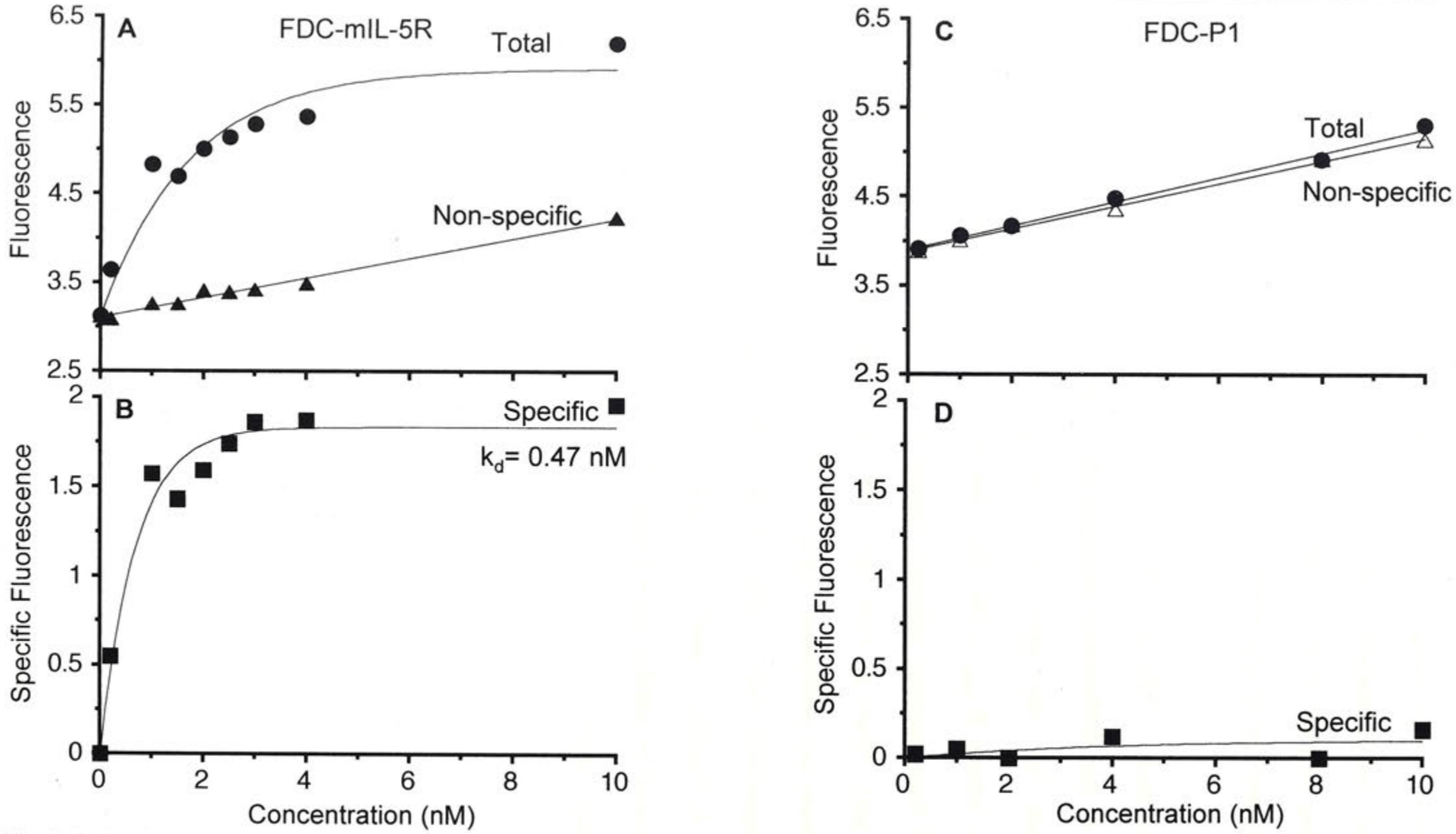


Fig. 3.4. Receptor binding of mIL-5~Sfx using a flow cytometry-based binding assay.
Typical receptor binding curves are shown for FDC cells expressing the mIL-5 receptor (Panels A and B) and FDC-P1 cells lacking the mIL-5 receptor system (Panels C and D). The data were obtained from cytometric profiles as shown in Figures 3.2 and 3.3 and plotted as the geometrical mean of the fluorescence versus the concentration of mIL-5~Sfx. The curve for total binding was obtained in the presence of fluorescently labelled ligand, while the non-specific curve was acquired in the presence of additional unlabelled ligand in 100-fold excess (Panels A and C). The data were plotted for specific binding as the difference between the total and non-specific binding (Panels B and D, and fitted for the function $B(x)=B_{max}(1-e^{-kx})$, where B_{max} is the maximal binding, k is the rate constant, and x is the concentration of ligand in nM.

3.3 The mIL-5 NLS1⁻ mutant form of mIL-5 exhibits drastically reduced binding affinity to its receptor

Using the flow cytometric approach for measuring the ligand-receptor interaction as described above, the binding capability of the mIL-5 NLS1⁻ mutant derivative was examined. Receptor binding of mIL-5 NLS1⁻ mutant was investigated using FDC-mIL-5R cells exposed to the fluorescently labelled mIL-5 NLS1⁻ mutant derivative in the range of 0.2-10 nM and analysed by flow cytometry as described in Section 3.2. In contrast to wild type mIL-5, the mIL-5(NLS1⁻) mutant derivative showed drastically reduced specific binding to FDC-mIL5R cells (Figs. 3.5 and 3.6), indicating that the binding affinity to the mIL-5 receptor system is severely affected by the mutation in the proximal arm of the putative NLS region of mIL-5.

This would explain the results obtained for *in vitro* receptor piggy back experiments, in which the mIL-5(NLS1⁻) mutant derivative failed to cotranslocate the soluble mIL-5 receptor subunits to the nucleus (Calanni, 1997). It also suggests that the inability of this IL-5 mutant form to stimulate the production of eosinophil colonies in bone marrow cultures may have been due to a deficiency in high affinity binding to the IL-5 receptor, rather than specific interference with nuclear targeting of this NLS mutant derivative.

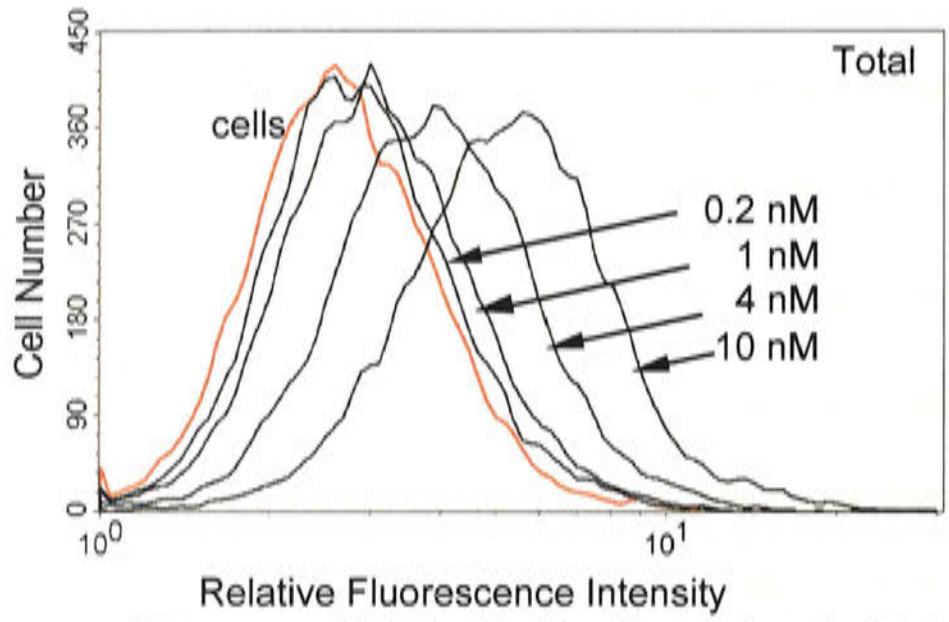


Fig. 3.5. Fluorescence histogram of FDC-mIL-5R cells, incubated with increasing concentrations of mIL-5(NLS1⁻)~Sfx.

Cells of the mouse myeloid progenitor cell line FDC-P1 stably transfected with the cDNA for mIL-5Rα were incubated with increasing concentrations of mIL-5(NLS1⁻)~Sfx at 4°C as described in Section 2.12.1 of Materials and Methods.

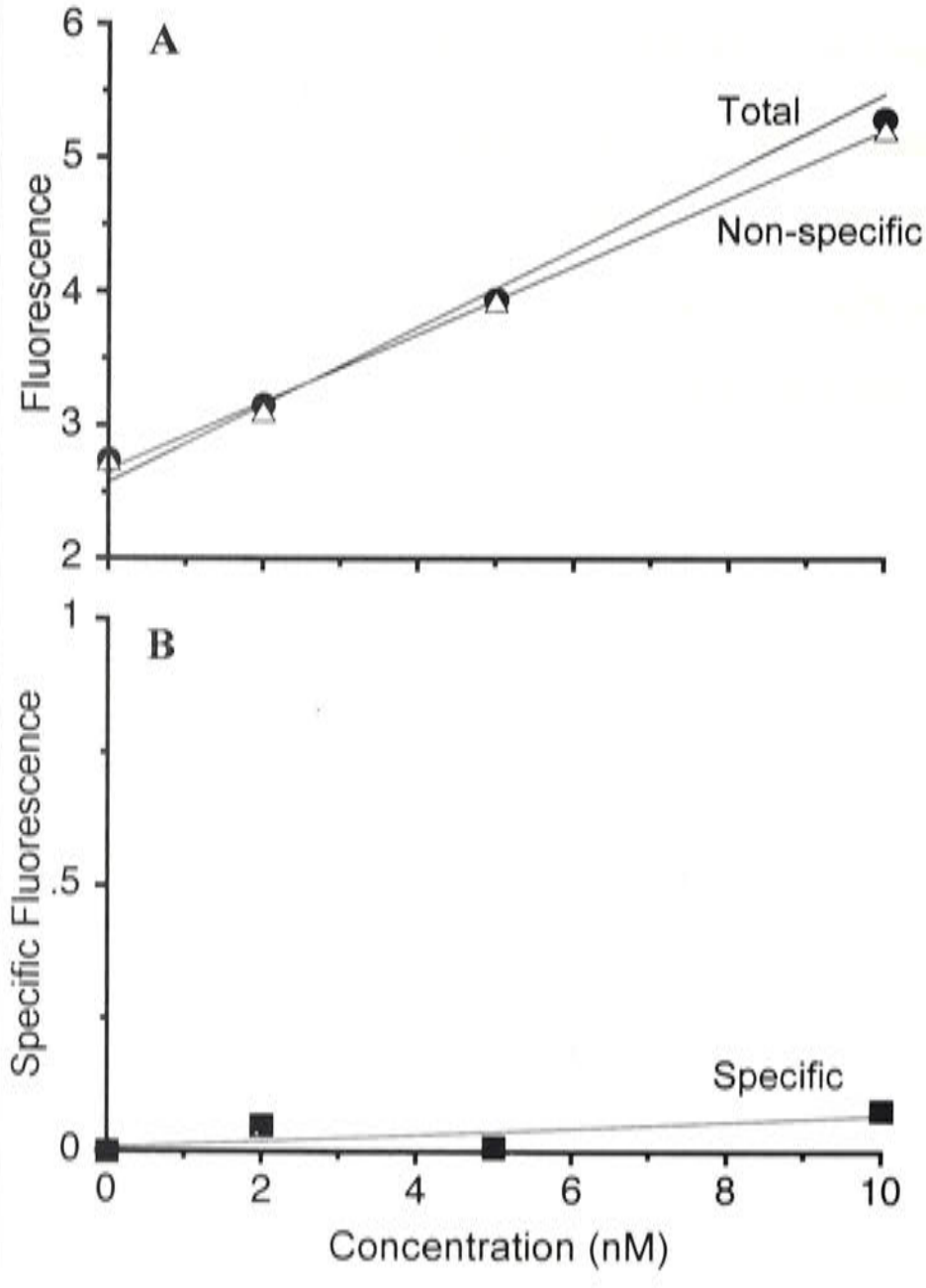


Fig. 3.6. Receptor binding of mIL-5~Sfx (NLS1⁻) mutant.

Typical receptor binding curves are shown for FDC-mIL-5R cells. The data were obtained from cytometric profiles as in Fig. 3.5 and plotted as the geometrical mean of the fluorescence versus concentration of the NLS1⁻mutant derivative of mIL-5~Sfx.

The curve for total binding was obtained in the presence of fluorescently labelled ligand, while the non-specific curve was acquired in the additional presence of unlabelled ligand in 100-fold excess (Panel A).

The data were replotted for specific binding as the difference between the total and non-specific binding (Panel B).

The data were fitted for the function $B(x)=B_{max}(1-e^{-kx})$, where B_{max} is the maximal level of specific binding, k is the rate constant, and x is the concentration of ligand in nM.

3.4 Construction of a human IL-5-NLS3⁻ mutant-β-Gal fusion

Previous investigations of IL-5-mediated piggyback nuclear import of the IL-5 receptor subunits indicated that the distal arm of the IL-5 NLS-like region (NLS2) was not important for nuclear translocation of the ligand-receptor complex *in vitro* (Calanni, 1997). These results implied that the putative NLS region of IL-5 might not be a conventional bipartite NLS, defined as two basic clusters linked by a 10 to 12 amino acid spacer sequence (Robbins *et al.,* 1991). Therefore, the involvement of a third cluster (NLS3) between the distal and proximal cluster (Fig. 3.7.) in nuclear transport of IL-5 was investigated.

One method to assess the functionality of an NLS is to test its ability to target the large *E. coli* β-galactosidase enzyme (476 kDa) to the nucleus (Silver, 1984; Rihs, 1991). Previously, it has been shown that the IL-5wt-NLS was indeed capable of targeting β-galactosidase to the nucleus, while the hIL-5NLS1⁻ mutant was unable to do so (Jans *et al.*, 1997a). The new hIL-5 NLS3⁻-β-Gal construct was derived from hIL-5wt-NLS-β-Gal by mutation of the NLS3 region. Lysine 102 was substituted by asparagine, and lysine 104 was replaced by alanine to obtain the mutant sequence shown in Fig. 3.7.

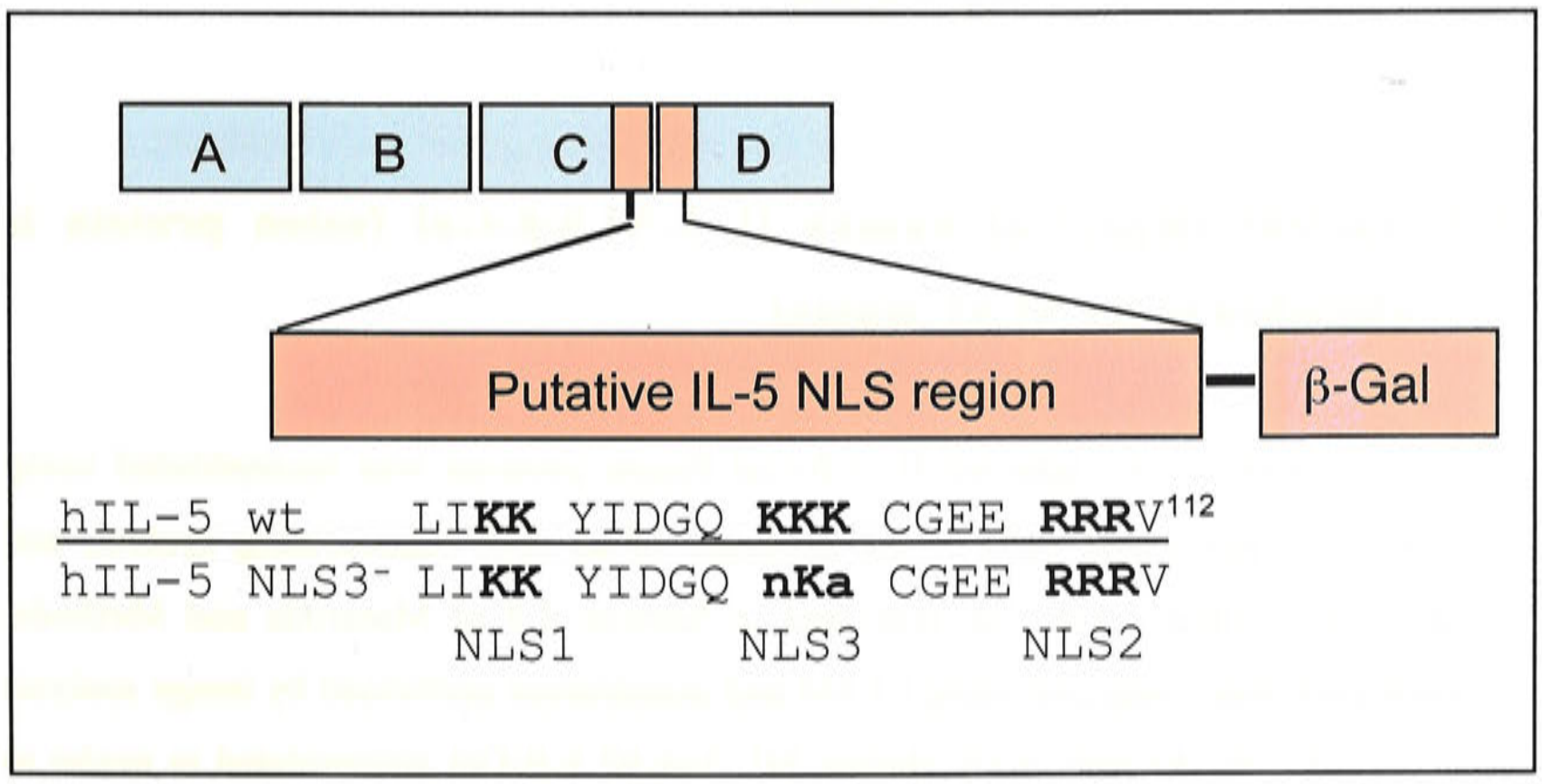


Fig 3.7. The hIL-5-NLS3⁻-β-Gal fusion protein. The sequences for hIL-5(wt), and the hIL-5(NLS3⁻) mutant form are indicated in the single letter amino acid code. Basic residues and mutations therein are indicated in bold.

The plasmid expressing a hIL-5-NLS3$^-$-β-Gal fusion protein was generated by oligonucleotide site-directed, single strand mutagenesis of plasmid pPR2hIL5wt (Jans *et al.,* 1997a). The primer hNLS3NKA (see Table 2.6) with both mutation and selection characteristics was utilised to generate the site-specific mutations, whereby the unique restriction site Bal I of the plasmid template was removed to serve as the basis for selection against non-mutated plasmids. The plasmid pPR2hIL5wt was heat denatured, the primer annealed and T4 DNA polymerase used to synthesise the new strand. The mixture was then treated with the restriction enzyme *Bal I* to linearize all non-mutated plasmids. The digested mixture consisting of mutated and wild type DNA was used to transform the repair-defective *E. coli* strain NM522 (mutS) (see Table 2.3) mutated in double-stranded DNA repair. Plasmid DNA was then isolated from the transformed cells and subjected to a second round of Bal I restriction enzyme selection to increase the proportion of mutated plasmids. The mixture was transformed into the *E. coli* strain MC 1060 (see Table 2.3) and plated on LBA plates. Constructs were screened by restriction analysis using Stu I, and a positive clone of the plasmid pPR2hNKA (see Table 2.5) sequenced to confirm its identity. The hIL-5-NLS3$^-$-β-Gal protein was expressed in *E. coli* MC1060 cells and purified by affinity chromatography as described in Section 2.4 of Materials and Methods. It was subsequently labeled with IAF (see Section 2.3.4) for nuclear transport studies.

3.5 Nuclear import of human IL-5-NLS-β-Gal fusion protein is abolished in the NLS3$^-$ mutant

Nuclear import of IAF-labelled IL-5-β-Gal fusion proteins was reconstituted using mechanically perforated cells in the presence of an ATP-regenerating system, and exogenously added cytosol as described in Section 2.9 of Materials and Methods. Fluorescence was visualized using CLSM and quantitation performed by image analysis (see Section 2.9). As previously shown, hIL-5wt-NLS-β-Gal accumulated in nuclei to levels approximately 3-fold those in the cytoplasm (Table 3.1), with half maximum accumulation achieved within c. 9 minutes. Nuclear accumulation hIL-5wt-NLS-β-Gal was found to be dependent on both ATP and exogenous cytosol, in identical fashion to

the NLS-containing T-ag-β-Gal fusion protein (Fig. 3.8, Table 3.1). Similarly, the non-hydrolysable GTP analog GTPγS inhibited transport (Fig. 3.8, Table 3.1). The results thus implied that hIL-5wt-NLS-β-Gal localizes in the nucleus through an ATP- and cytosolic factor-dependent pathway inhibitable by GTP analogs, accumulation thus likely to be an NLS-dependent process dependent on importin and Ran/TC4.

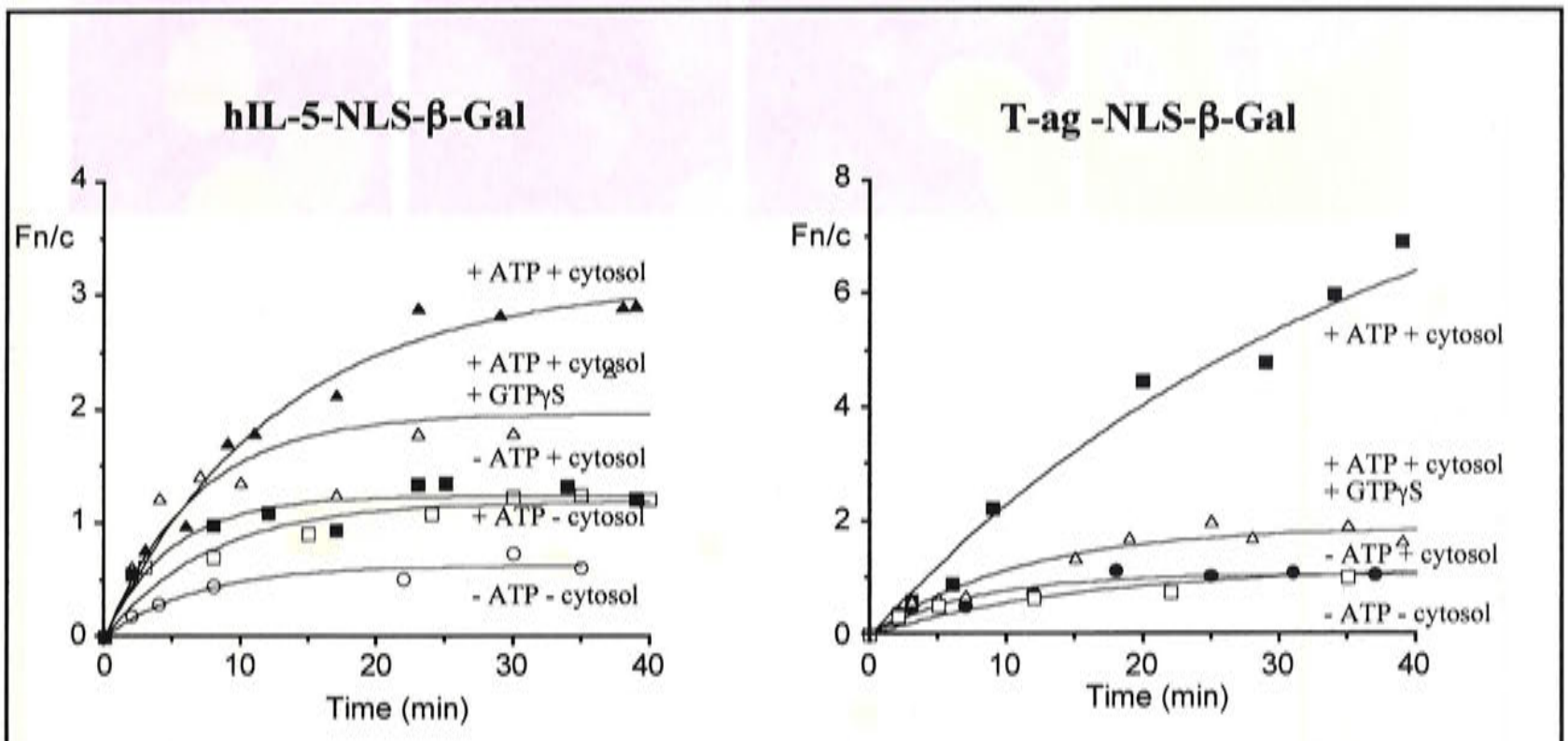


Fig. 3.8. Nuclear transport kinetics of the hIL-5-NLS-β-galactosidase fusion protein *in vitro*.
Nuclear import was reconstituted in mechanically perforated HTC cells. Experiments were carried out in the presence and absence of exogenous cytosol and an ATP-regenerating system or the non-hydrolysable GTP analog GTPγS (in the presence of exogenous cytosol and an ATP-regenerating system), as indicated. Where cytosol was not used, an equivalent concentration of BSA was included. Results are compared to those for an NLS-containing T-ag-β-galactosidase fusion protein. Data were fitted for the function $Fn/c = Fn/c_{max} (1-e^{-kt})$, where Fn/c_{max} is the maximal level of nuclear accumulation, k is the rate constant, and t is time in minutes. Pooled data are presented in Table 3.1.

Nuclear exclusion of hIL-5-NLS1$^-$-β-Gal (Fn/c_{max} of 0.48) was observed as previously described (Jans *et al.*, 1997a), consistent with NLS1 (see Fig. 3.7) being integral to NLS function. The hIL-5-NLS3$^-$-β-Gal fusion protein was also completely excluded from the nucleus of mechanically perforated HTC cells, with an Fn/c_{max} of approximately 0.5 (Fig. 3.9; Table 3.1).

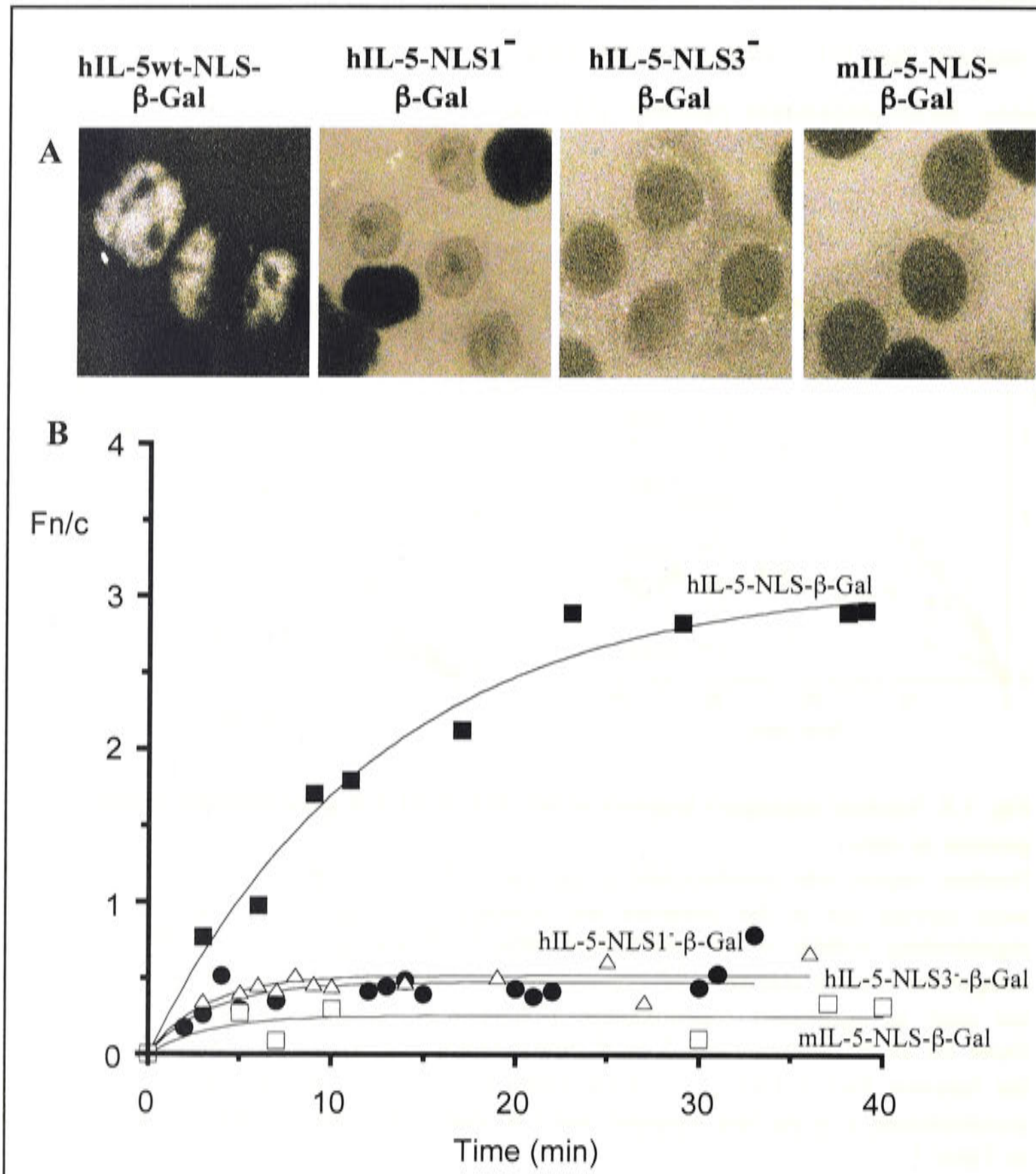


Fig. 3.9. Nuclear import of IL-5-NLS-β-galactosidase fusion proteins *in vitro*.
Nuclear import was reconstituted in mechanically perforated HTC cells. (A) CLSM images of nuclear accumulation of NLS-β-Galactosidase fusion proteins after 40 min in the presence of cytosol and an ATP-regenerating system. (B) Nuclear import kinetics from a single typical experiment performed as in (A) for NLS-β-Galactosidase fusion proteins in the presence of cytosol where each data point represents at least three separate measurements for each of Fn (nuclear fluorescence), Fc (cytoplasmic fluorescence), and background fluorescence. Data were fitted for the function $Fn/c = Fn/c_{max} (1-e^{-kt})$, where Fn/c_{max} is the maximal level of nuclear accumulation, k is the rate constant, and t is time in minutes. Pooled data are presented in Table 3.1.

Table 3.1. Nuclear import kinetics of IL-5 and IL-5-β-galactosidase fusion protein derivatives compared to those of SV40 T-ag-β-galactosidase fusion proteins in mechanically perforated HTC cells.

Protein [b]	Nuclear Import Parameter [a]		
	Fn/c_{max}	$t_{1/2}$	n
hIL-5wt-NLS-β-Gal (1 x IB)	0.63 ± 0.06	N.D. [c]	1
hIL-5wt-NLS-β-Gal (+ ATP/ + cytosol)	3.11 ± 0.23 [d]	8.7 ± 0.9	7
hIL-5wt-NLS-β-Gal (+ ATP/ - cytosol)	1.19 ± 0.07 [d]	5.3 ± 0.5	1
hIL-5wt-NLS-β-Gal (- ATP/ + cytosol)	1.24 ± 0.11 [d]	3.5 ± 0.6	1
hIL-5wt-NLS-β-Gal (+ ATP/ + cytosol/ + GTPγS)	1.96 ± 0.12 [d]	4.6 ± 0.3	1
T-ag-NLS-β-Gal (1 x IB)	1.19 ± 0.24	12 ± 0.8	2
T-ag-NLS-β-Gal (+ ATP/ + cytosol)	9.67 ± 0.32	25 ± 0.9	8
T-ag-NLS-β-Gal (+ ATP/ - cytosol)	N.D.	N.D.	
T-ag-NLS-β-Gal (- ATP/ + cytosol)	1.07 ± 0.16	N.D. [c]	2
T-ag-NLS-β-Gal (+ ATP/ + cytosol/ + GTPγS)	1.89 ± 0.4	7.5 ± 0.4	3
T-ag-NLS-β-Gal (+ ATP/ + cytosol + CHAPS)	1.14 ± 0.15	N.D. [c]	2
T-ag-NLS-β-Gal (+ ATP/ + cytosol + anti Impβ)	1.34 ± 0.07	N.D. [c]	2
N1N2-NLS-β-Gal (+ ATP/ + cytosol)	5.9 ± 0.28	17.32± 0.58	2
hIL-5-NLS1$^-$-β-Gal (+ ATP/ + cytosol)	0.48 ± 0.29	N.D. [c]	2
hIL-5-NLS3$^-$-β-Gal (+ ATP/ + cytosol)	0.52 ± 0.04	N.D. [c]	2
mIL-5wt-NLS-β-Gal (+ ATP/ + cytosol)	0.26 ± 0.04	N.D. [c]	2

[a] Results represent the mean ± S.E. (n indicated) from data fitted to the function $Fn/c = Fn/c_{max}\ (1 - e^{-kt})$, where Fn/c_{max} is the maximal level of accumulation at steady state in the nucleus, k is the rate constant, and t is time in min. $t_{1/2}$ represents the time to reach half maximal accumulation and is calculated by the equation 0.693/k.

[b] All experiments were performed in the presence of factors as indicated; where cytosol was not used, BSA (45mg/ml) was included

[c] N.D., not able to be determined due to low nuclear accumulation; IB- intracellular buffer (see Table 2.1)

[d] SE was determined from the curve fit

The best-characterised monopartite NLS is that of T-ag (residues 126-132; PKKKRKV), while the archetypal bipartite NLS is that of nucleoplasmin (Dingwall *et al.*, 1982; Robbins *et al.*, 1991). T-ag and *X. laevis* phosphoprotein N1N2 NLSs are potent targeting signals, and β-Gal fusion proteins containing these NLSs were therefore used as control molecules. Nuclear levels of 10- and 6-fold those in the cytoplasm were observed for T-ag-β-Gal and N1N2-β-Gal respectively (Fig. 3.10; Table 3.1).

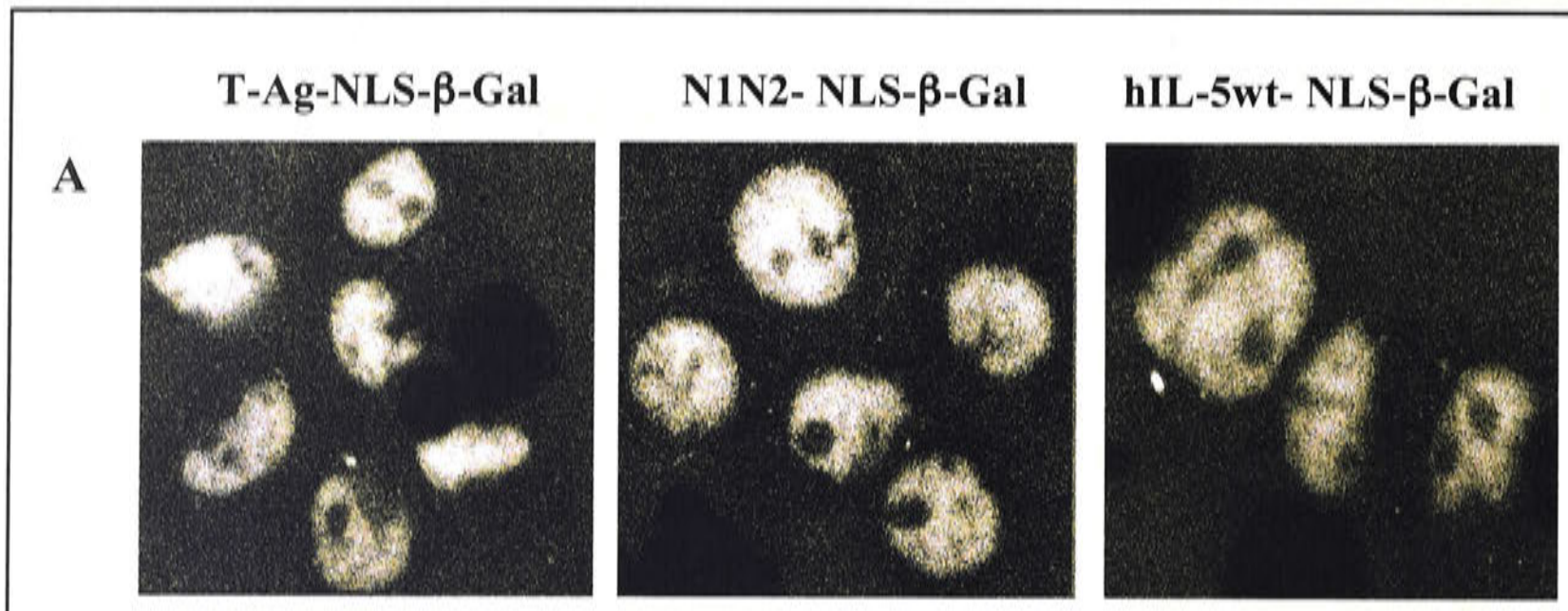


Fig. 3.10. Nuclear import of different NLS-β-galactosidase fusion proteins *in vitro*.
Nuclear import was reconstituted in mechanically perforated HTC cells. Experiments were carried out in the presence of exogenous cytosol and an ATP-regenerating system.
(A) CLSM images of nuclear accumulation of NLS-β-galactosidase fusion proteins.
(B) Nuclear import kinetics from a single typical experiment performed as in (A) for NLS-β galactosidase fusion proteins. Pooled data are presented in Table 3.1.

Taken together the effects of the IL-5 NLS1$^-$ and NLS3$^-$ mutations, both basic clusters appear to be necessary for nuclear translocation of β-galactosidase.

In order to assess the importance of the hIL-5 NLS3 sequence in IL-5 function, site directed mutagenesis was used to alter the hIL-5 sequence to introduce the identical mutation to that used in the hIL-5-NLS3$^-$-β-Gal fusion protein construct. However, the NLS3-deficient hIL-5 could not be expressed in the baculovirus system. It therefore proved impossible to assess the nuclear targeting ability on the part of the NLS3-deficient hIL-5 in vitro, or to examine which IL-5 signalling functions were impaired. The basis of the lack of expression of the NLS3-deficient hIL-5 derivative was not determined directly, but was probably attributable to the introduced amino acid changes resulting in structural instability of the mutated IL-5 molecule. Similar observations were made before for an NLS1-deficient hIL-5 derivative (Jans and Young, unpublished data).

3.6 Nuclear import is abolished in a mouse IL-5-NLS(wt)-β-Gal fusion protein

Both the hIL-5 NLS1 and NLS3 regions appeared to be necessary for targeting by the hIL-5 NLS of the heterologous β-Galactosidase protein to the nucleus. However, when the hIL-5 NLS is compared with the mIL-5 NLS, a marked difference in the NLS3 region becomes apparent. Glycine at position 100 is exchanged to arginine, while the basic residue lysine ^{102} is replaced by glutamic acid (Fig. 3.11).

The fact that the NLS region of human IL-5 was able to target the heterologous protein hIL-5-NLS-β-Gal to the nucleus raised the question of whether the mouse sequence was also able to translocate β-Gal to the nucleus. To analyze if the changes in the mouse IL-5-NLS region had an impact on the capability of the region to translocate β-Gal to the nucleus, a mouse IL-5-NLS-β-Gal fusion protein construct was designed (see Fig. 3.11).

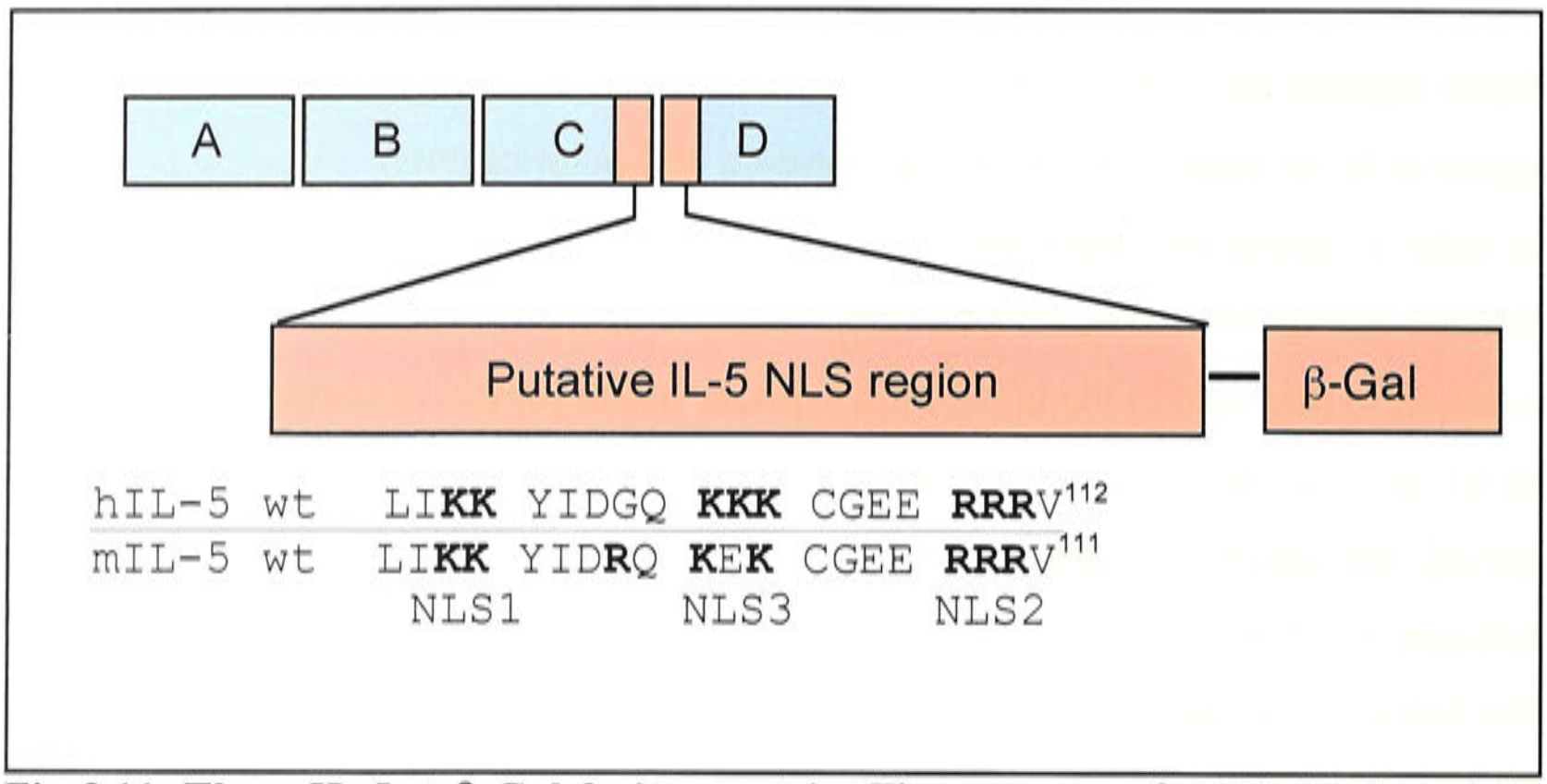


Fig 3.11. The mIL-5wt-β-Gal fusion protein. The sequences for hIL-5(wt), and the mIL-5wt form are indicated in the single letter amino acid code. Basic residues are indicated in bold.

To obtain the wild type sequence for mouse IL-5-NLS, lysine 103 and glycine 100 of the hIL-5-NLS(wt) sequence were substituted by glutamic acid and arginine respectively, whereby the mIL-5wtNLS-β-Gal construct was derived from hIL-5wt-NLS-β-Gal by double stranded mutagenesis of the pPR2hIL-5wt plasmid (see Section 2.2.14 of Materials and Methods). The primers mKEKsense and mKEKanti (see Table 2.6) were utilized to generate the site-specific mutations, whereby the unique restriction site Bal I of the plasmid template was removed to serve as the basis for screening against non-mutated plasmids. The two synthetic oligonucleotide primers, each complementary to opposite strands of the vector, were extended during temperature cycling by native PfuTurbo DNA polymerase under conditions described in Section 2.2.14 of Materials and Methods. On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks was generated. After temperature cycling, the product was treated with 10 units of the restriction endonuclease Dpn I for 1 hour at 37°C to digest the parental DNA template and select for the synthesized DNA containing mutations. The resulting mix was digested with the restriction enzyme Dpn I, which is specific for methylated and hemi-methylated DNA. The nicked vector DNA incorporating the desired mutations was then transformed into the competent *E.coli* strain Top 10 F' (see Table 2.3) and cells grown overnight on LBA plates. Constructs were verified by colony PCR (see Section 2.2.16) and screened by restriction analysis

using Bal I and a positive clone plasmid pPR2mKEK sequenced to confirm its identity. The mIL-5wt-β-Gal protein was expressed in *E. coli* MC1060 cells and purified by affinity chromatography as described for the other β-Gal fusion proteins.

Nuclear import was assessed *in vitro* using mechanically perforated HTC cells in the presence of an ATP-regenerating system and exogenously added cytosol. The mIL-5wt-NLS-β-Gal fusion protein was completely excluded from the nucleus of mechanically perforated HTC cells. Maximum nuclear accumulation levels were approximately 0.3 relative to the cytoplasm (Fig 3.9; Table 3.1), comparable to those of hIL-5-NLS1$^-$-β-Gal and hIL-5-NLS3$^-$-β-Gal, indicating that the mouse putative NLS region is not sufficient to target β-Gal to the nucleus in the *in vitro* nuclear transport assay. This raised the question of the role of the NLS region in nuclear import of IL-5 itself.

3.7 Expression and labelling of mIL-5 and mutant derivatives

In order to investigate the mechanistic basis of nuclear transport of IL-5 in the context of the entire IL-5 molecule, mIL-5 and NLS mutant derivatives were utilized for *in vitro* nuclear transport studies (Fig. 3.12).

Beside an NLS1$^-$ mutant, a monomeric form of mIL-5 was generated. In the native form of IL-5, the short loop between helices C and D prevents unimolecular folding of helix D into a functionally obligate structural motif (see Fig. 1.2 in Section 1.1.5). By lengthening this loop, an insertional, monomeric form of IL-5 can be created that shows biological activity similar to that of native IL-5 (Dickason and Huston, 1996). The introduction of the eight amino acid spacer between K^{101} and E^{102} separates the two lysines K^{101} and K^{103}, which makes the monomeric form comparable to the IL-5 NLS3$^-$ mutant.

mIL-5 and NLS mutant derivatives were expressed and purified by the Cytokine Molecular Biology and Signalling Group, JCSMR as described in Section 2.5 and fluorescently labelled as described in Section 2.3.4 of Materials and Methods.

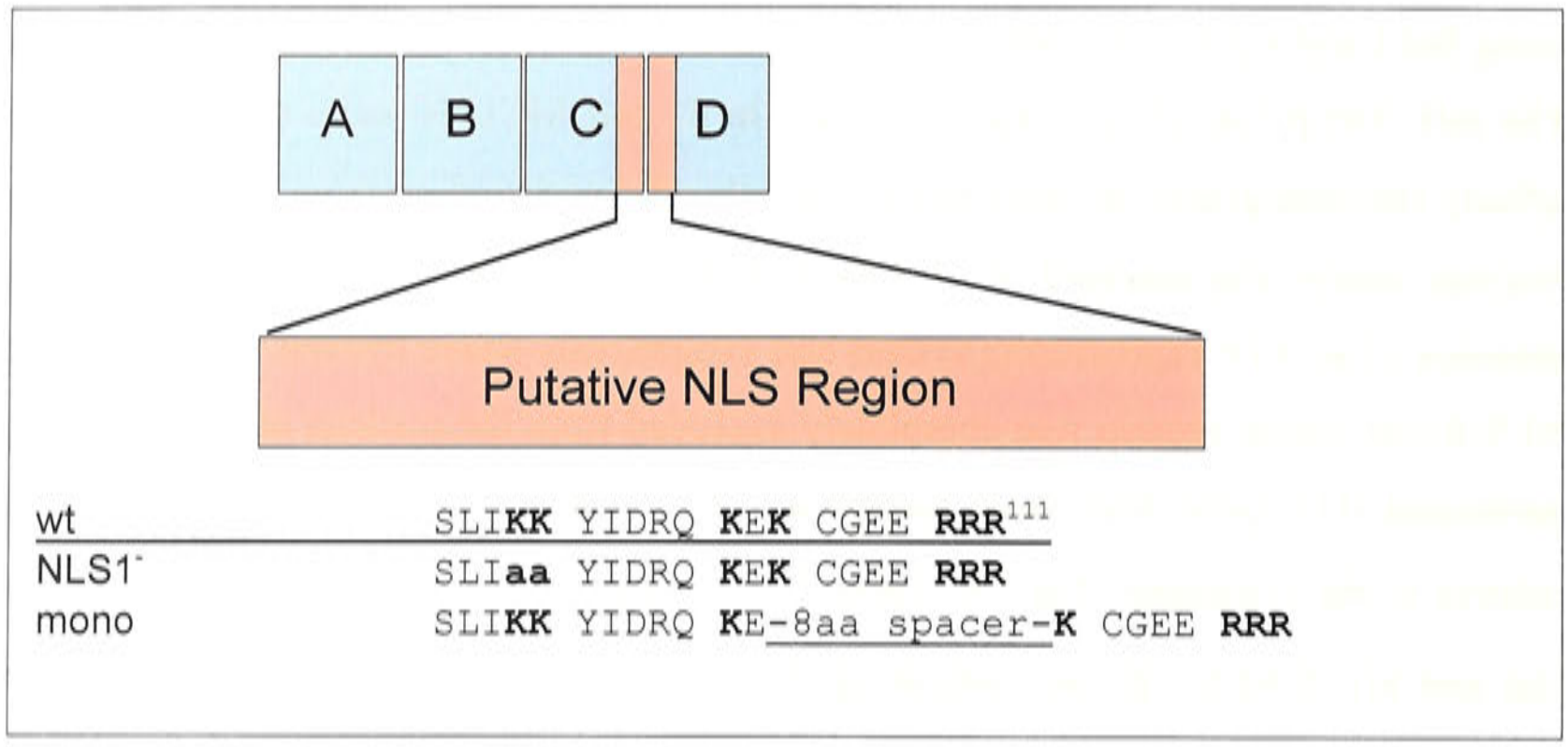


Fig. 3.12. mIL-5 and its NLS mutant derivatives. The sequences for mIL-5(wt), the mIL-5(NLS1$^-$) mutant form and the monomeric mIL-5 form are indicated in the single letter amino acid code. Basic residues proposed to be crucial components of the NLS are indicated in bold. aa, amino acids

3.8 Nuclear import of IL-5 *in vitro* does not require cytosolic components

In order to carry out kinetic measurements of IL-5 nuclear transport, baculovirus-expressed Sfx-labelled IL-5 and NLS mutant derivatives thereof were used. The fluorescent derivatives retained biological activity in proliferation assays using mouse myeloid progenitor FDC-mIL-5R cells.

The nuclear import kinetics of these SFX-labelled derivatives was measured in *in vitro* as described in the previous sections.

In vitro nuclear transport assays with mIL-5 and its NLS mutant derivatives showed that wild type IL-5 as well as the NLS derivatives accumulated rapidly and strongly in the nucleus of HTC cells (Fig 3.12, Panels A and B; Table 3.2). Specifically, mIL-5wt accumulated in the nucleus to levels approximately 2.4-fold those in the cytoplasm, with half maximal accumulation achieved within 3 minutes ($t_{1/2}$ 3.15). The level of accumulation of the NLS mutants was similar to that of wild type. For the NLS1 mutant, nuclear accumulation was 2.4-fold higher than in the cytoplasm, with the half maximum accumulation achieved within c. 2 minutes ($t_{1/2}$ 1.92). The monomeric NLS3 mutant form, showed 2.2-fold nuclear accumulation over cytoplasm, with half-maximum accumulation attained at 1.5 minutes (Fig. 3.13).

Table 3.2. *In vitro* nuclear import kinetics for mIL-5 and NLS mutant derivatives.

PROTEIN [b]	Nuclear Import Parameter [a]		
	Fn/c $_{max}$	$t_{1/2}$	n
hIL-5-Sfx (1 x IB)	2.31 ± 0.06	0.49 ± 0.6	4
hIL-5-Sfx (+ ATP/ + cytosol)	2.09 ± 0.19	0.39 ± 0.36	6
hIL-5-Sfx (+ ATP/ + cytosol/ + GTPγS)	1.90 ± 0.03	0.63 ± 0.5	2
mIL-5-Sfx (1 x IB)	2.35 ± 0.06	3.15 ± 0.28	6
mIL-5-Sfx (+ ATP/ + cytosol)	2.73 ± 0.43	1.9 ± 0.5	2
mIL-5-Sfx (+ ATP/ - cytosol)	2.15 ± 0.07	1.3 ± 0.5	2
mIL-5-Sfx (- ATP/ + cytosol)	2.30 ± 0.38 [d]	1.8 ± 0.7	1
mIL-5-Sfx (+ ATP/ + cytosol/ + GTPγS)	1.90 ± 0.10 [d]	0.76 ± 0.4	1
mIL-5(NLS1$^-$)	2.40 ± 0.12	1.92 ± 0.16	3
mIL-5(mono)	2.20 ± 0.21	1.5 ± 0.39	3
mIL-5 + WGA	1.20 ± 0.04	N.D. [c]	3
mIL-5 + CHAPS	1.0 ± 0.24	N.D. [c]	2
mIL-5 + importin β (full length)	1.17 ± 0.19	N.D. [c]	2
mIL-5 + importin β (1-380)	2.60 ± 0.33	5.78 ± 0.47	2
mIL-5 + importin α	2.26 ± 0.09 [d]	5.47 ± 0.14	1

[a] Results represent the mean ± S.E. (n indicated) from data fitted to the function $Fn/c = Fn/c_{max}\ (1 - e^{-kt})$, where Fn/c_{max} is the maximal level of accumulation at steady state in the nucleus, k is the rate constant, and t is time in min. $t_{1/2}$ represents the time to reach half maximal accumulation and is calculated by the equation
$0.693/k$.
[b] All experiments were performed in the presence of factors as indicated; where cytosol was not used, BSA (45mg/ml) was included
[c] N.D., not able to be determined due to low nuclear accumulation; IB- intracellular buffer (see Table 2.1)
[d] SE was determined from the curve fit

As mentioned in Section 3.5, NLS-dependent nuclear protein import *in vitro* is dependent on ATP. *In vitro* experiments were performed to investigate the dependence of mIL-5 nuclear import on ATP and cytosolic factors (Fig 3.14; Table 3.2). Maximal nuclear accumulation of mIL-5 was dependent of neither ATP nor exogenous cytosol, although the rate of nuclear import was increased by about 2-fold (Table 3.2), implying that nuclear import was accelerated by both ATP and cytosolic factors. GTPγS appeared to have little effect on nuclear import of mIL-5, in contrast to its strong inhibition of nuclear import of the NLS-containing T-ag β-Gal fusion protein (Fig.3.8; Table 3.1).
A clear difference was thus observed in the dependence of the mIL-5 nuclear import on ATP and cytosolic factors compared to hIL-5 NLS-β-gal dependent on both ATP and exogenous cytosol (Table 3.1; Fig. 3.8), in identical fashion to conventional NLS-containing β-Gal fusion proteins.

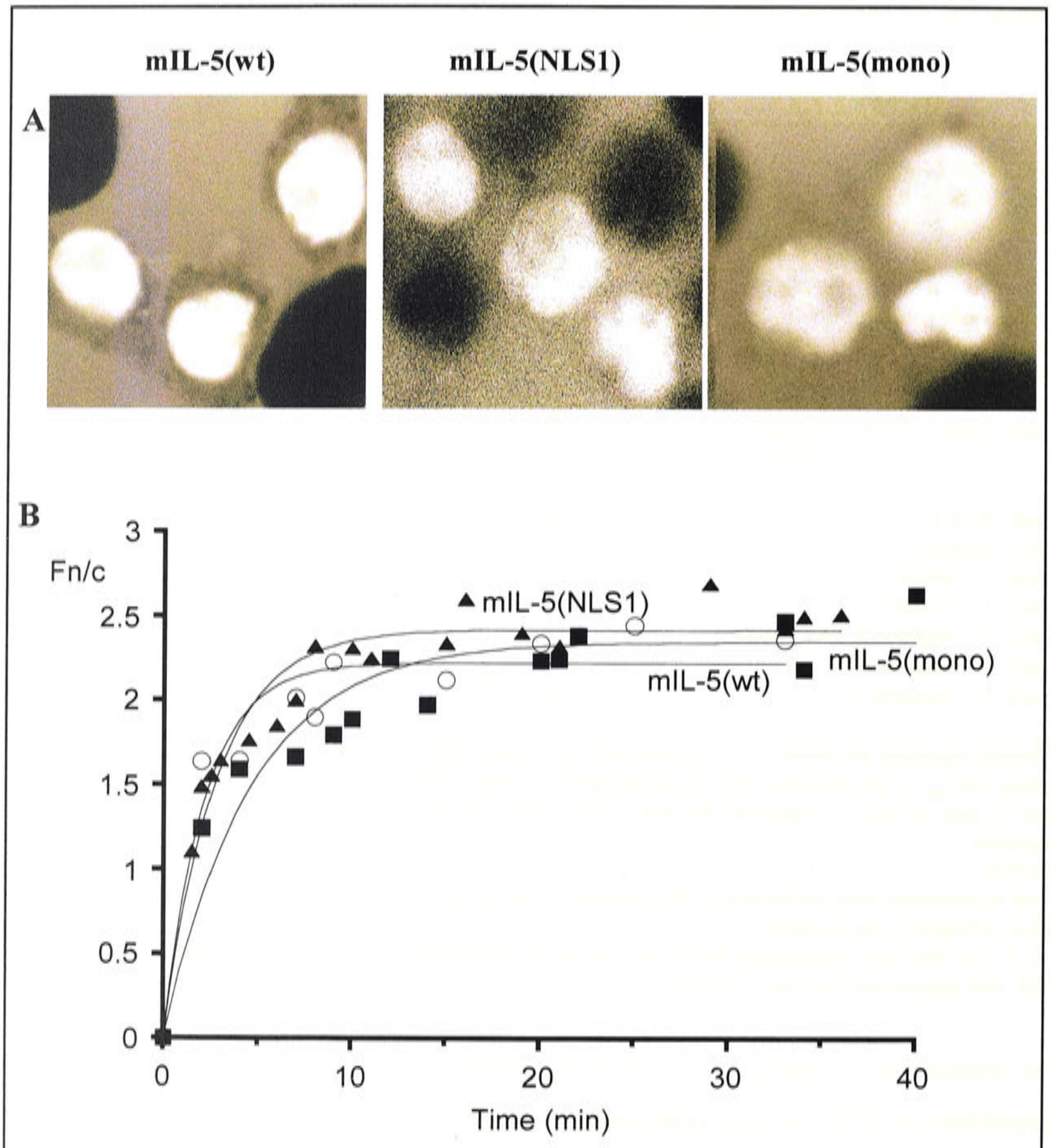


Fig. 3.13. Nuclear import of mIL-5 mutant derivatives *in vitro*.
Nuclear import was reconstituted in mechanically perforated HTC cells. (A) CLSM images of nuclear accumulation of mIL-5-Sfx and derivatives after 30 min in the absence of exogenous cytosol or ATP. (B) Nuclear import kinetics from a single typical experiment performed as in (A) for mIL-Sfx and derivatives in the absence of cytosol where each data point represents at least three separate measurements for each of Fn (nuclear fluorescence), Fc (cytoplasmic fluorescence), and background fluorescence. Data were fitted for the function $Fn/c = Fn/c_{max}(1-e^{-kt})$, where Fn/c_{max} is the maximal level of nuclear accumulation, k is the rate constant, and t is time in minutes. Pooled data are presented in Table 3.2.

Similarly to mIL-5, maximal nuclear accumulation of hIL-5 was not dependent of ATP or exogenous cytosol, and the rate of nuclear import was similar with and without transport factors (Fig. 3.14; Table 3.2). Besides, GTPγS appeared to have no effect on nuclear import of hIL-5.

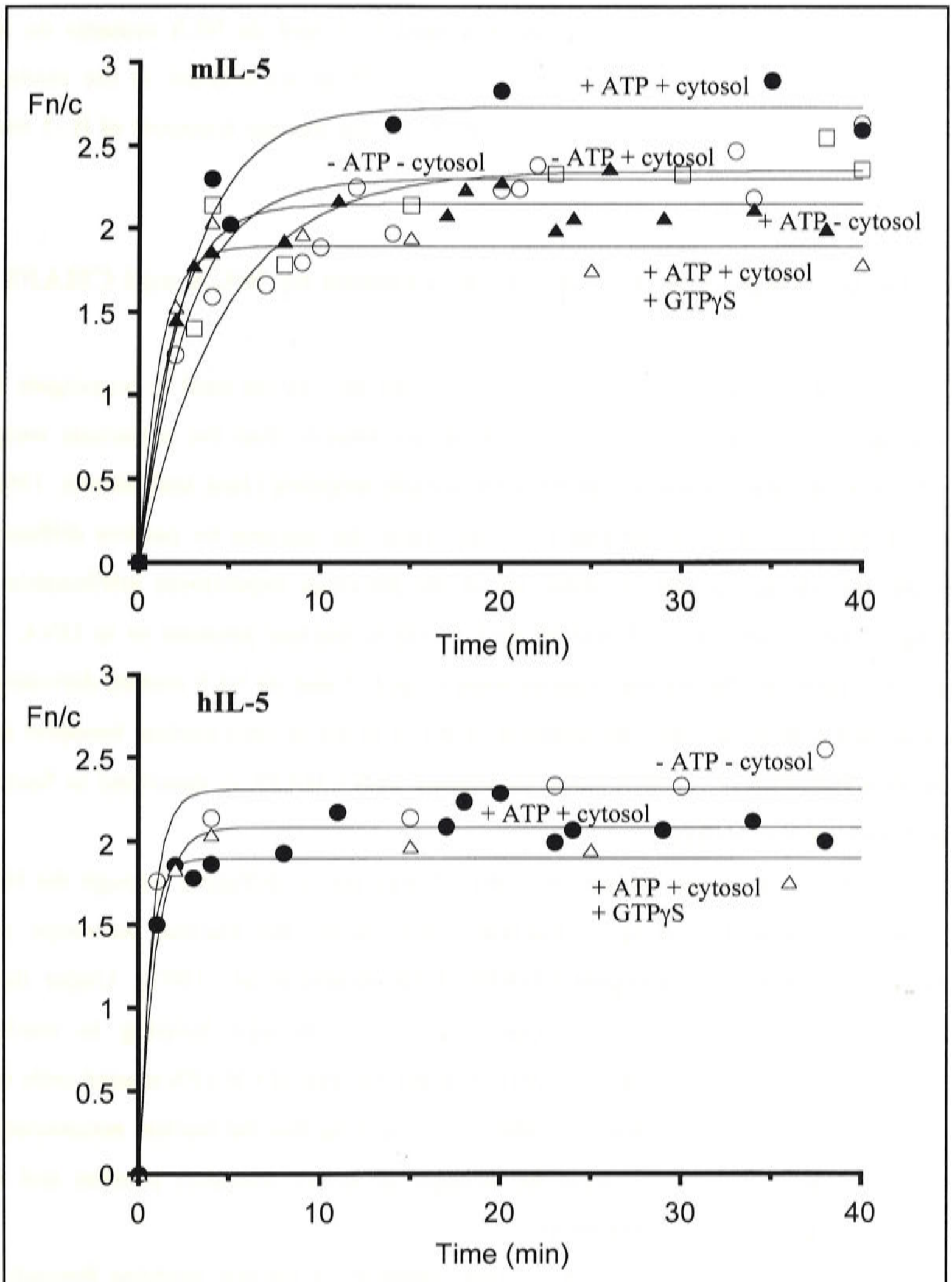


Fig. 3.14. Nuclear import of hIL-5 and mIL-5 *in vitro*.
Nuclear import was reconstituted in mechanically perforated HTC cells. Experiments were carried out in the presence and absence of exogenous cytosol and an ATP-regenerating system or the non-hydrolysable GTP analog GTPγS (in the presence of exogenous cytosol and an ATP-regenerating system), as indicated. Data were fitted for the function $Fn/c = Fn/c_{max} (1-e^{-kt})$, where Fn/c_{max} is the maximal level of nuclear accumulation, k is the rate constant, and t is time in minutes. Pooled data are presented in Table 3.2.

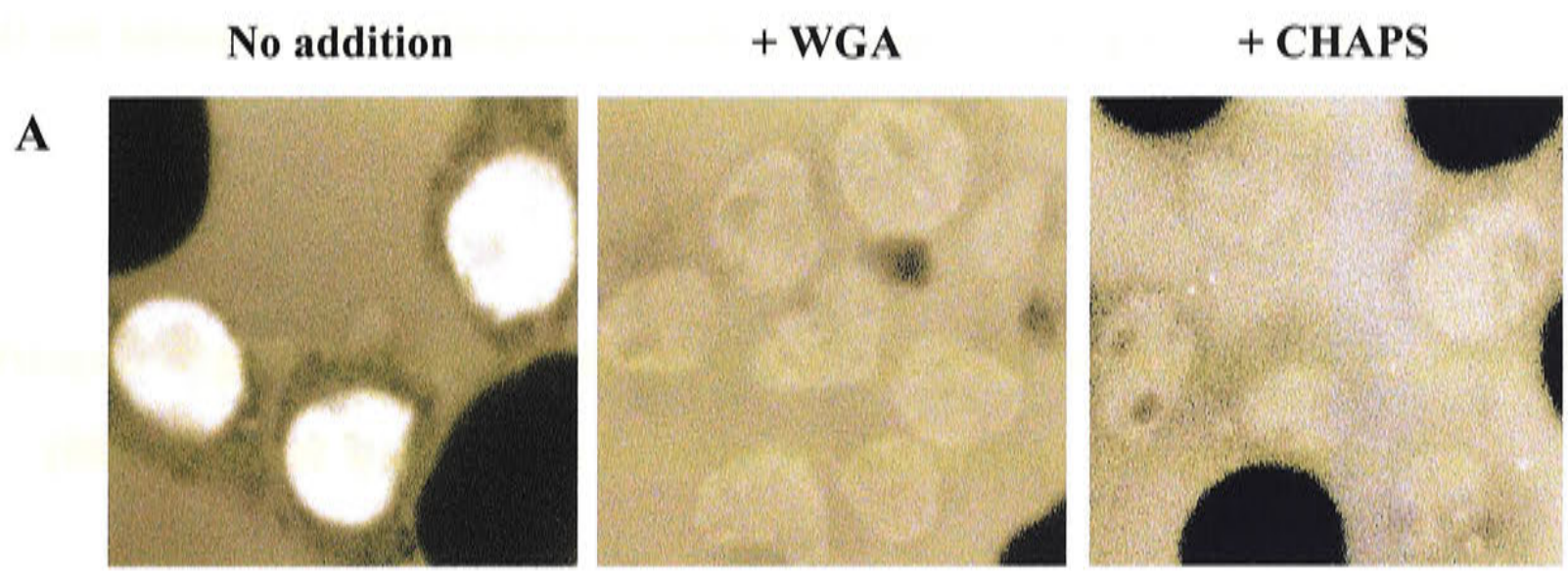
Thus, whereas hIL-5-NLS-β-Gal localizes in the nucleus through an ATP- and cytosolic factor-dependent pathway, baculovirus-expressed IL-5 and its NLS mutants do not require exogenously added cytosolic factors or ATP to accumulate in the nucleus, indicating that different mechanisms are responsible for nuclear transport of IL-5 itself and the IL-5-NLS-β-Gal fusion protein.

3.9 Nuclear import of IL-5 *in vitro* is inhibited by WGA and CHAPS

The recombinant protein mIL-5 and its NLS mutant derivatives used to investigate the nuclear targeting capability of IL-5 (~30 kDa) are smaller than the molecular weight cut-off (50 kDa) requirement for an NLS for nuclear targeting (Jans and Hübner, 1996). It would therefore be expected that IL-5 may enter the nucleus by passive diffusion, with the nuclear accumulation observed in the previous experiment attributable to binding of mIL-5 and its NLS mutant derivatives to nuclear proteins or to DNA. To clarify this question, the nuclear translocation of mIL-5 and its NLS mutant derivatives was analysed following either the addition of WGA to the *in vitro* nuclear transport mix or the permeabilisation of the nuclear membrane with CHAPS as described in Section 2.9 in Materials and Methods.

To test whether nuclear accumulation of mIL-5 was due to diffusion through the NPC into the nucleus and binding of nuclear components, the nuclear envelope was permeabilized using the detergent CHAPS (Efthymiadis *et al.*, 1997). Under these conditions, nuclear accumulation can only occur through binding to nuclear components. Nuclear accumulation of mIL-5 in the nucleus of CHAPS-treated cells was abolished (Fn/c 1, Fig. 3.15 Panel B; Table 3.2), implying that the nuclear accumulation of mIL-5 evident in Fig.3.12 occurred through an active transport process and not through binding to nuclear components.

The lectin WGA is known to block nuclear transport of nuclear proteins through its ability to bind O-linked glycoside N-acetyl-glucosamine moieties on FxFG nucleoporins (see Section 1.2.3). The essential role of these proteins in nuclear trafficking has been shown by the fact that depletion of WGA-binding nucleoporins from nuclei inhibits transport, with restoration of transport activity being achieved by readdition of the WGA-binding protein fraction (Finlay and Forbes 1990).



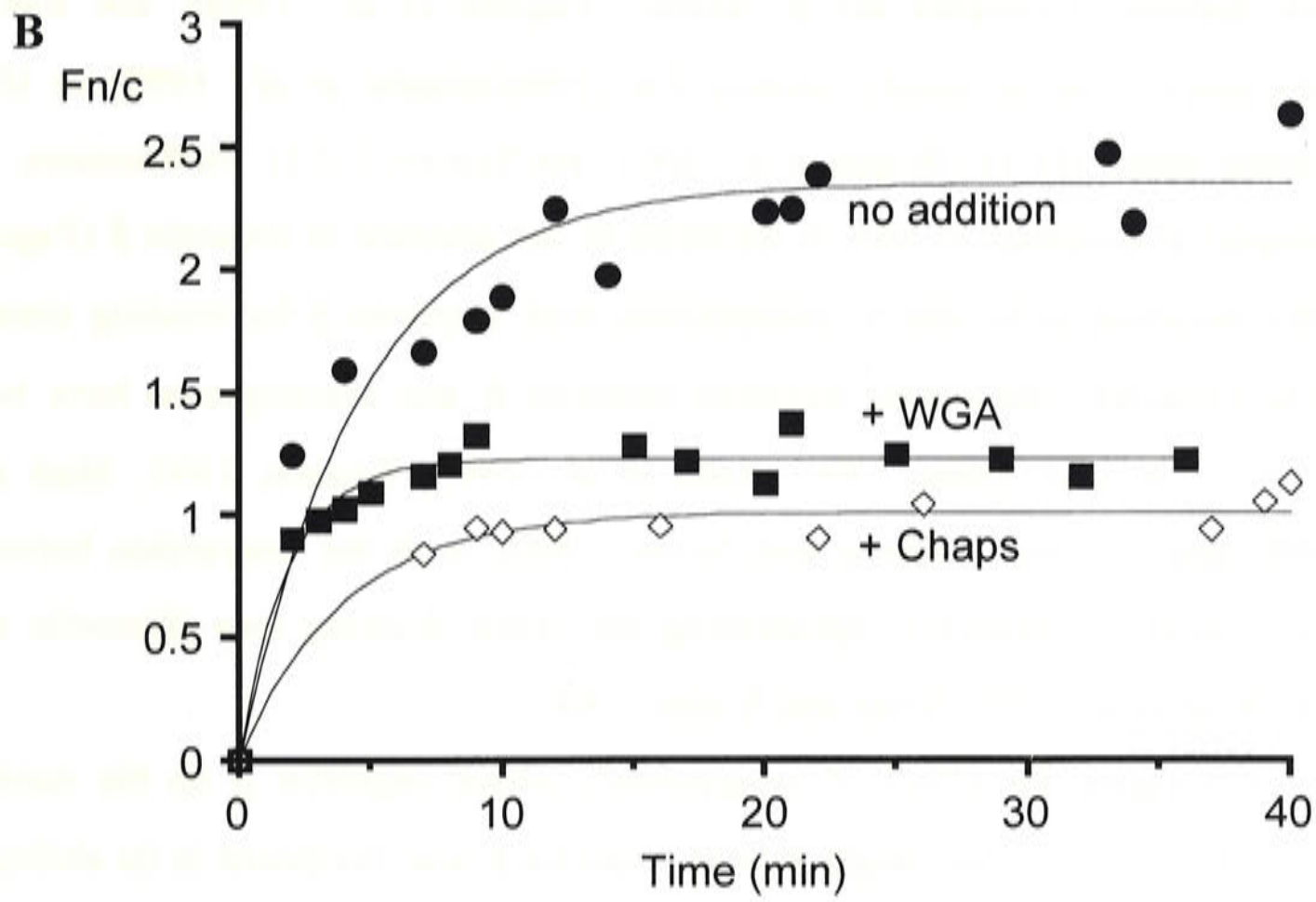


Fig. 3.15. Nuclear import of mIL-5-Sfx *in vitro*.
Nuclear import was reconstituted in mechanically perforated HTC cells. (A) CLSM images of nuclear accumulation of mIL-5-Sfx after 30 min in the absence or presence of CHAPS or WGA as indicated. (B) Nuclear import kinetics from a single typical experiment performed as in (A) for mIL-5-Sfx in the absence of cytosol with the additions as indicated where each data point represents at least three separate measurements for each of Fn (nuclear fluorescence), Fc (cytoplasmic fluorescence), and background fluorescence. Data were fitted for the function $Fn/c = Fn/c_{max} (1-e^{-kt})$, where Fn/c_{max} is the maximal level of nuclear accumulation, k is the rate constant, and t is time in minutes. Pooled data are presented in Table 3.2.

WGA drastically reduced nuclear accumulation of mIL-5, from original nuclear levels approximately 2.5-fold higher than in the cytoplasm to equal levels in the nucleus and cytoplasm (Fn/c 1.2, Fig. 3.15), indicating that nucleoporins were required for IL-5 nuclear transport which clearly therefore occurred through the NPC.

3.10 Nuclear import of IL-5 *in vitro* is inhibited by full length importin β, but not by a C-terminally truncated importin β form (1-380)

It is known that a number of proteins can translocate to the nucleus in an importin-independent fashion. Examples are β-catenin (Fagotto *et al.,* 1998), the human immunodeficiency virus accessory protein Tat (Efthymiadis *et al.,* 1998) or IFN-induced nuclear factor IFI 16 (Briggs *et al.,* 2001; see Section 1.2.5). Furthermore, the nuclear transport of β-catenin *in vitro* is inhibited by the addition of importin β (Fagotto *et al.,* 1998), believed to be due to competition with importin β for binding sites at nucleoporins. Detailed interactions between importin β and nucleoporins have been shown *in vitro* (Chi and Adam, 1997; Radu *et al.,* 1995; Rexach, 1995; Shah and Forbes, 1998) and *in vivo* (Damelin and Silver, 2000), with the interaction between nup358 and importin β possibly representing the initial docking step (Damelin and Silver, 2000; Rout *et al.,* 2000; Ryan and Wente, 2000).

In order to investigate the effect of exogenously added importin β on the nuclear transport of mIL-5 *in vitro*, full length human importin β was compared in its ability to inhibit nuclear transport of IL-5 with a truncated form spanning residues 1-380. Full length importin β was able to drastically reduce nuclear accumulation of mIL-5 to levels equal that of cytoplasm (Fn/c 1.17, Fig. 3.16), implying that importin β does compete with IL-5 for binding sites at the nuclear pore complex. In contrast, neither importin α nor the truncated version of importin β had an effect on the nuclear accumulation of mIL-5 (Fig. 3.16), suggesting that this truncated form may not compete for the same NPC-sites as importin β.

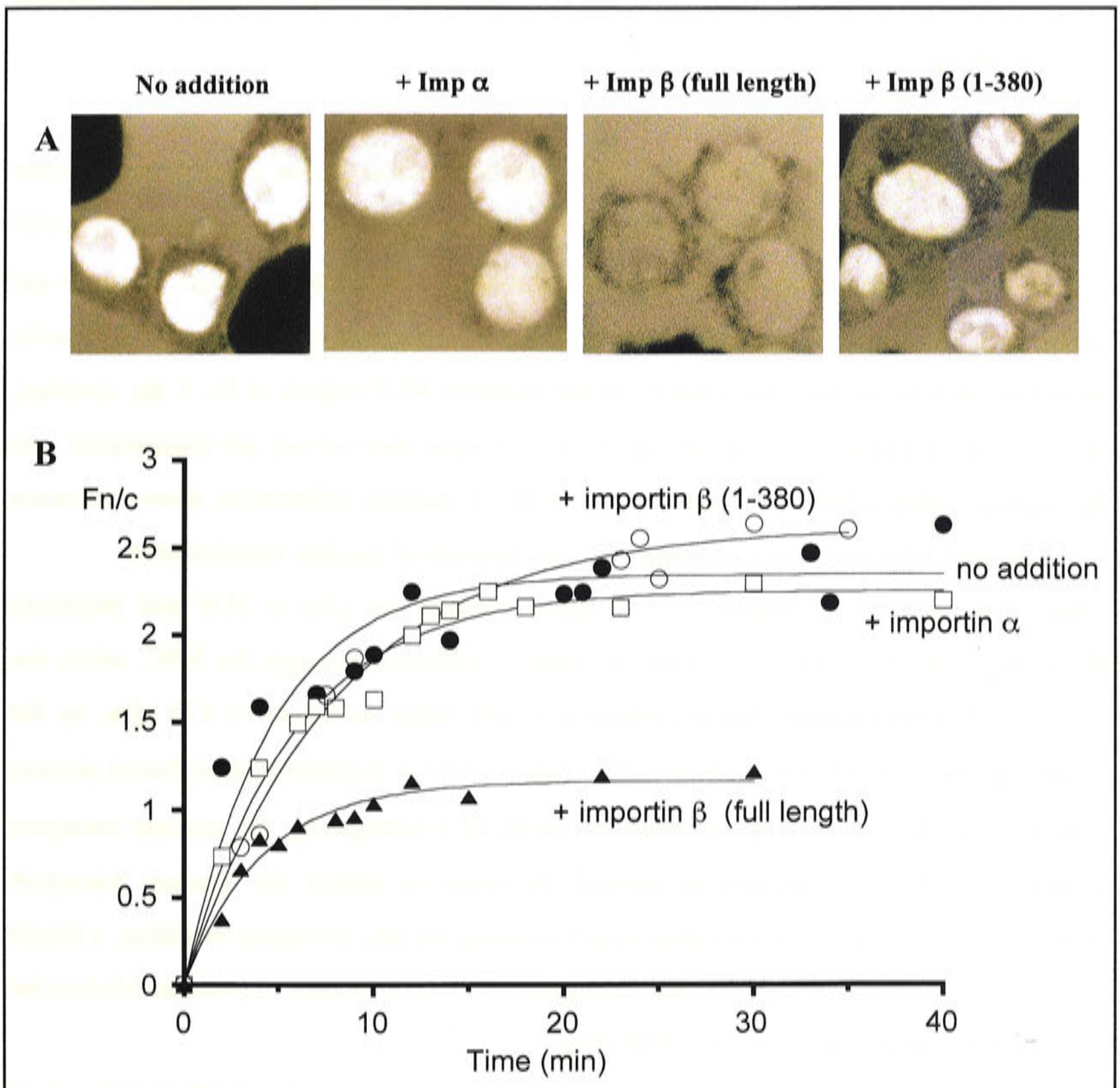


Fig. 3.16. Inhibition of nuclear import of mIL-5-Sfx by importin β *in vitro*.
Nuclear import was reconstituted in mechanically perforated HTC cells. (A) CLSM images of nuclear accumulation of mIL-5-Sfx after 30 min in the absence or presence of full length, C-terminally truncated importin β (100 pM) or importin α (100 pM) as indicated. (B) Nuclear import kinetics from a single typical experiment performed as in (A) for mIL-5 in the absence of cytosol with the additions as indicated where each data point represents at least three separate measurements for each of Fn (nuclear fluorescence), Fc (cytoplasmic fluorescence), and background fluorescence Data were fitted for the function $Fn/c = Fn/c_{max} (1-e^{-kt})$, where Fn/c_{max} is the maximal level of nuclear accumulation, k is the rate constant, and t is time in minutes. Pooled data are presented in Table 3.2.

3.11 Construction, expression and labelling of an IL-5-HSA fusion protein

The experimental results for the IL-5 NLS β-galactosidase fusion proteins are in clear contrast to those for baculovirus-expressed IL-5 with respect to the requirement for ATP and cytosolic components for nuclear accumulation. Nuclear transport of the β-gal fusion proteins seems to depend on the addition of exogenously added ATP, cytosolic factors as well as on the functionality of the putative NLS region of IL-5. By contrast, baculovirus-expressed IL-5 as well as its NLS mutant derivatives are transported into the nucleus independently of those factors, with no marked differences shown between wild type and NLS mutants with regards to the kinetics of nuclear translocation.

Clearly, baculovirus expressed IL-5 is approximately 30 kDa in MW and therefore below the "cut-off" value of 50 kDa for passive diffusion through the NPC, while the IL-5 NLS β-galactosidase fusion protein is a very large molecule of 476 kDa, by far exceeding the "cut-off" value of the NPC. The IL-5 NLS β-galactosidase fusion protein thus is not a very physiological model in terms of investigating the nuclear transport properties of IL-5 or sequences thereof. In order to assess the nuclear transport capabilities of IL-5 in a system better approximating the physiological situation, a single chained hIL-5 dimer construct was designed by David Mann, Cytokine Molecular Biology and Signalling Group, JCSMR (Fig. 3.17).

The hIL-5_{sc}-HSA fusion protein has several advantages compared to the β-galactosidase fusion protein construct. First, it contains the complete sequence of hIL-5, which in the single chain form has been shown to be fully functional (Li *et al.*, 1996). Secondly, the molecular weight of the HSA fusion protein is above the cut off value for passive diffusion through the NPC, but, by comparison with the IL-5 NLS β-gal fusion protein, does not exceed the size of a possible ligand-receptor complex (approx. 350 kDa), bearing in mind the observation that IL-5 is capable of piggybacking its soluble receptor subunits to the nucleus *in vitro* (Jans *et al.*, 1997b).

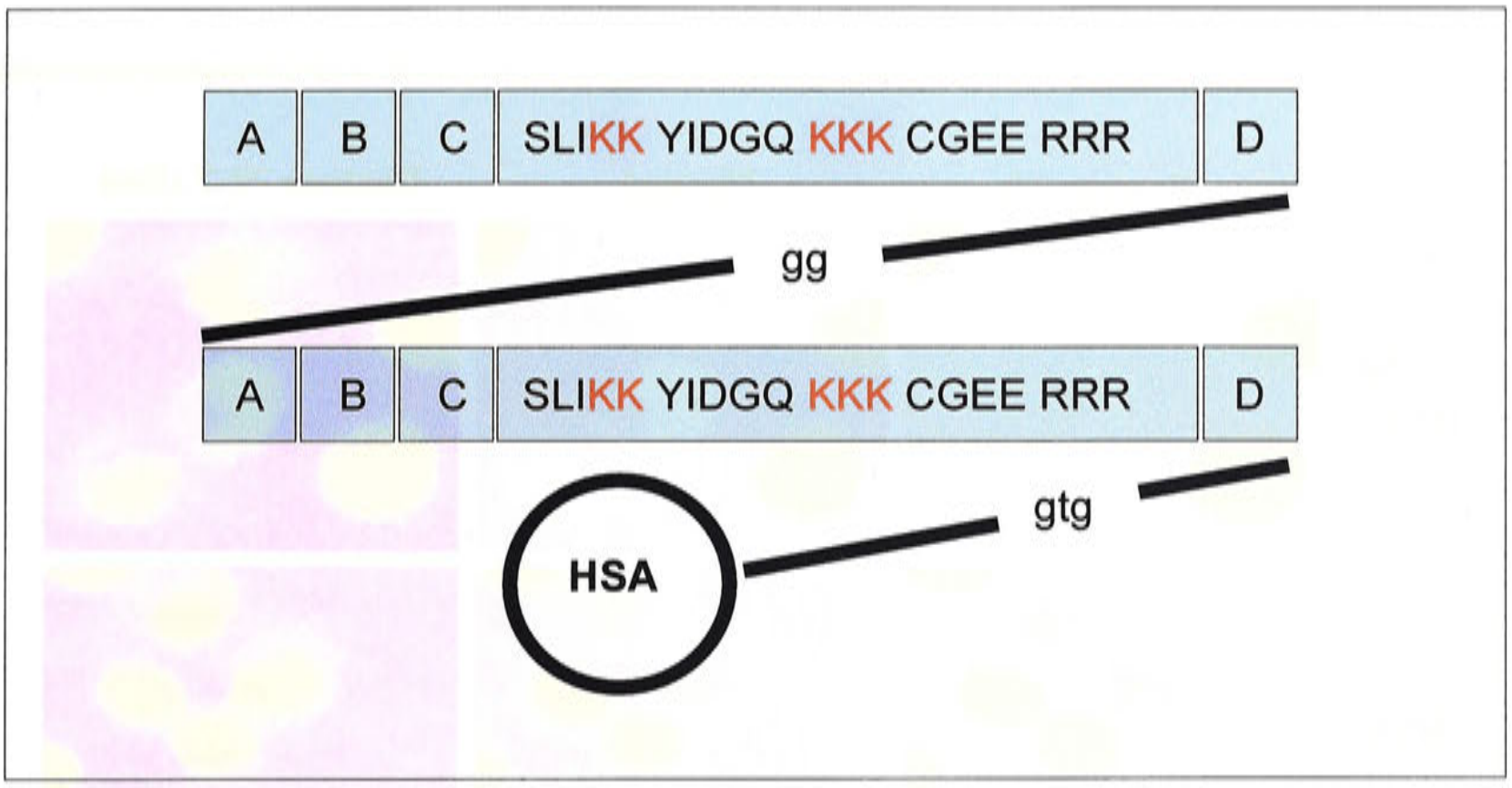
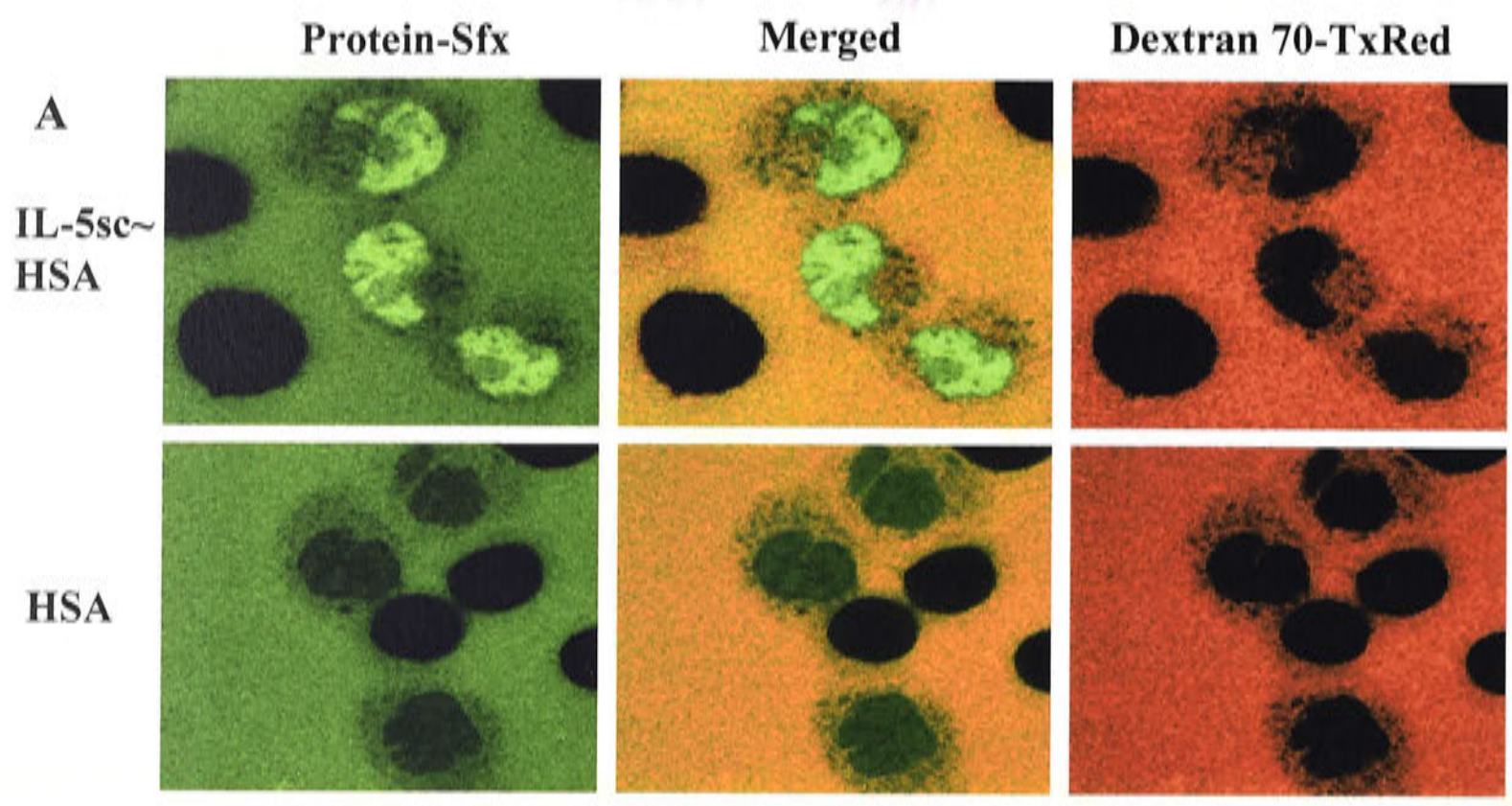


Fig. 3.17. Schematic representation of the human IL-5 single chain HSA fusion protein construct (hIL-5_{sc}-HSA). A gene encoding a single chain form of hIL-5 dimer fused to HSA was constructed by linking the two hIL-5 chain coding regions with a Gly-Gly linker, and linking the hIL-5_{sc} to HSA with a Gly-Thr-Gly linker. Expression of this gene using a baculovirus system in Sf9 insect cells yielded a biologically active hIL-5 single chain HSA fusion protein (hIL-5_{sc}-HSA).

3.12 Nuclear import of the 93kDa hIL5_{sc}-HSA fusion protein *in vitro* does not require ATP or exogenously added cytosolic factors

Nuclear import of the hIL-5_{sc}-HSA fusion protein was assessed *in vitro* using mechanically perforated HTC cells. *In vitro* nuclear transport assays with hIL-5_{sc}-HSA showed that it accumulated rapidly in the nucleus of HTC cells (Fig 3.18, Table 3.3), to levels approximately 2.5-fold higher than those in the cytoplasm (Fn/c 2.72).

Half maximal accumulation was achieved within 3 minutes ($t_{1/2}$ 3.3), which is slower than that of hIL-5 (30 kDa) alone (see Fig. 3.13, Table 3.2), likely because the molecular weight of IL-5_{sc}-HSA fusion protein (93 kDa) is 3 times greater. In contrast, HSA-Sfx was completely excluded from the nucleus (Fn/c 0.59), indicating that nuclear accumulation of hIL-5_{sc}-HSA was not due to the HSA moiety itself (Fig. 3.18, Panel B). A control 70kDa Dextran molecule was unable to diffuse into the nucleus (Fig. 3.18, Panel A), showing that the nuclear membrane was intact.



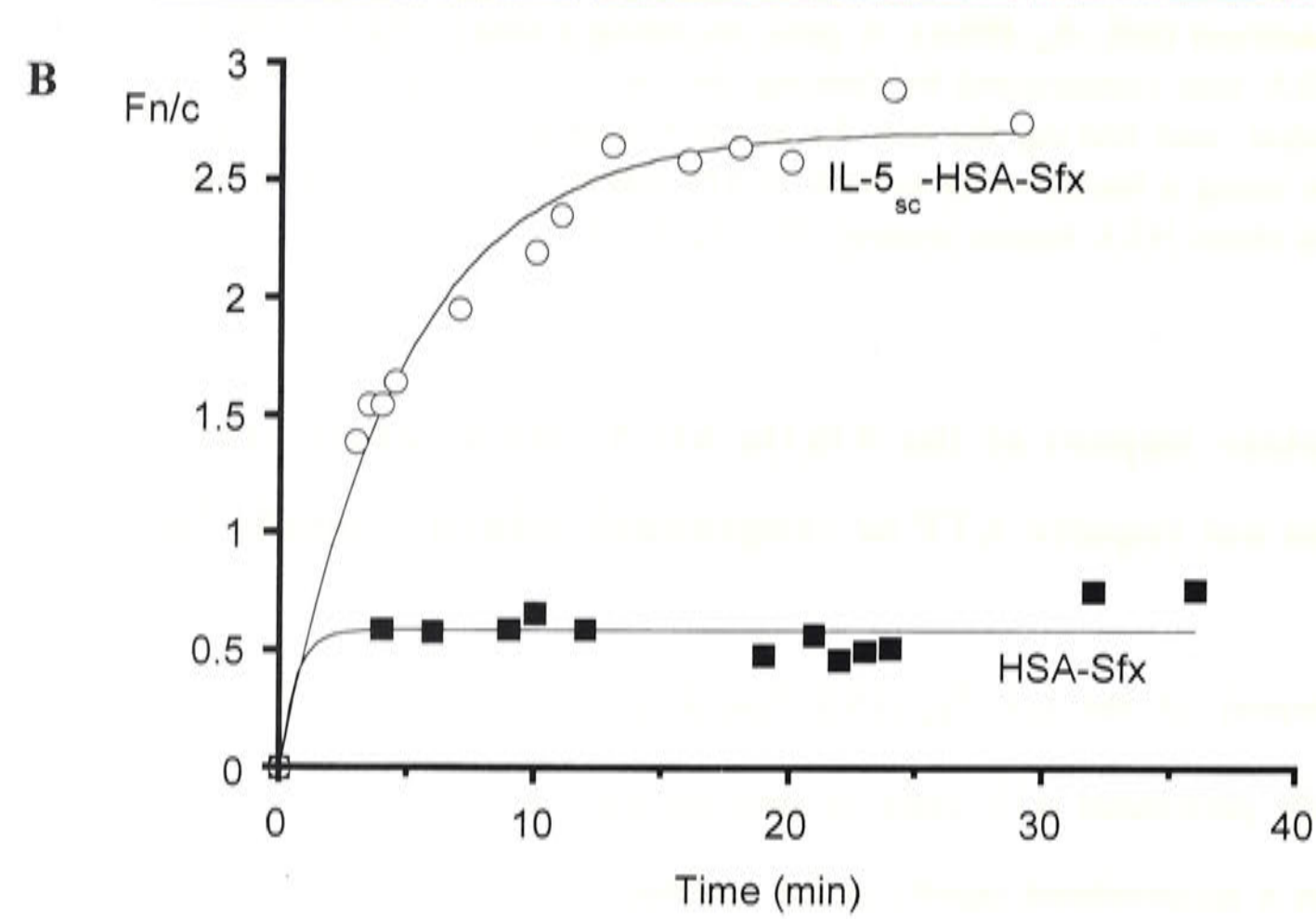


Fig. 3.18. Nuclear import of IL-5_{sc}-HSA *in vitro*.
Nuclear import was reconstituted in mechanically perforated HTC cells. (A) CLSM images of nuclear accumulation of IL-5sc-HSA-Sfx compared to HSA-Sfx after 30 min in the absence of exogenous cytosol or ATP. (B) Nuclear import kinetics from a single typical experiment performed as in (A) for HSA in the absence of cytosol where each data point represents at least three separate measurements for each of Fn (nuclear fluorescence), Fc (cytoplasmic fluorescence), and background fluorescence. Data were fitted for the function $Fn/c = Fn/c_{max}(1-e^{-kt})$, where Fn/c_{max} is the maximal level of nuclear accumulation, k is the rate constant, and t is time in minutes. Pooled data are presented in Table 3.3.

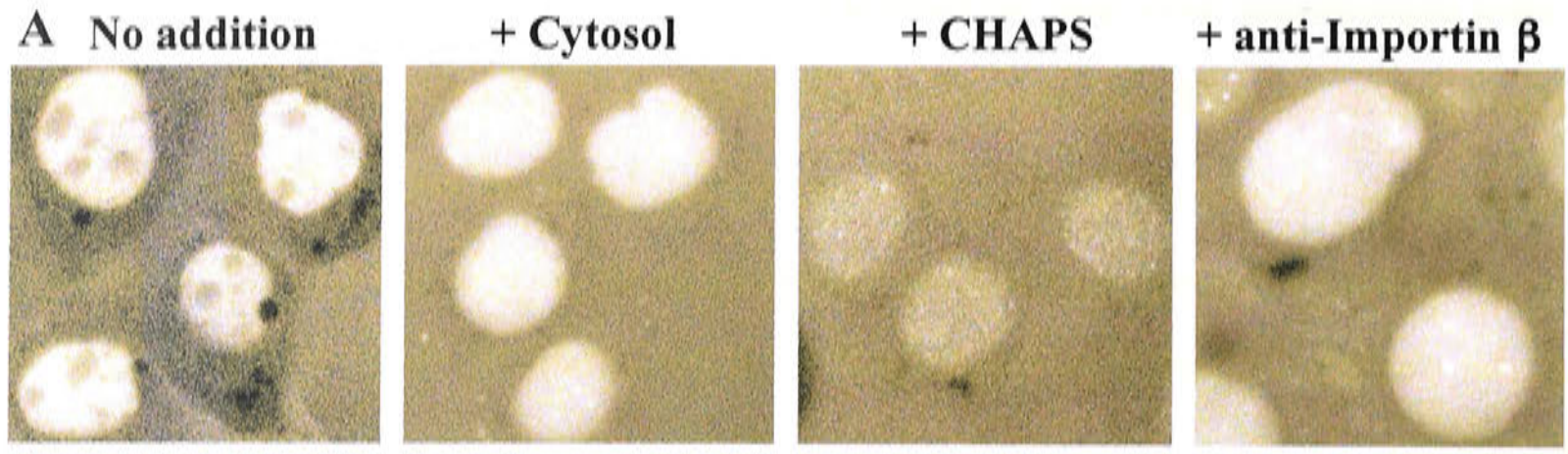
In vitro experiments were also performed to investigate the dependence of hIL-5_{sc}-HSA nuclear import on ATP and cytosolic factors (Fig. 3.19, Panel B; Table 3.3). Maximal nuclear accumulation of hIL-5_{sc}-HSA was independent of both ATP and exogenous cytosol, although the rate was slightly increased in their presence ($t_{1/2}$ 1.26), indicating that the nuclear import was accelerated to some extent by both ATP and cytosolic components.

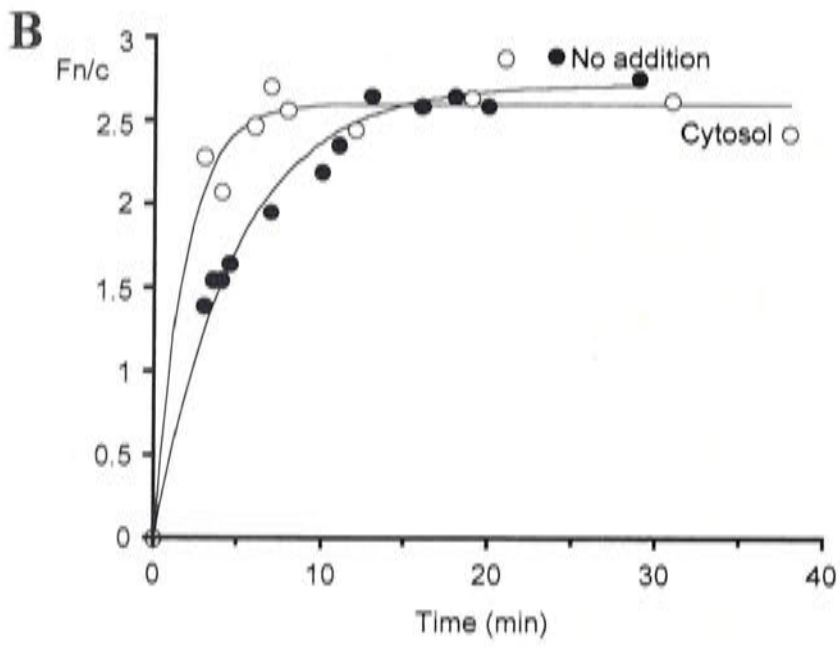
Table 3.3. In vitro nuclear import kinetics for hIL-5_{sc}-HSA.

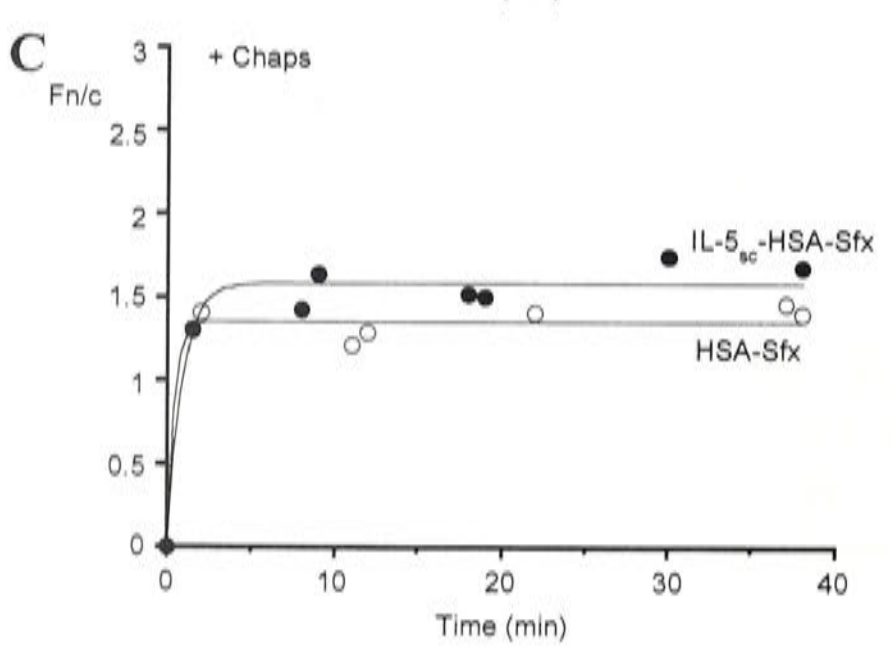
Protein	Nuclear Import Parameter [a]		
	Fn/c $_{max}$	**$t_{1/2}$**	**n**
hIL-5_{sc}-HSA	2.7 ± 0.07	3.46 ± 0.48	6
HSA	0.59 ± 0.05	N.D. [b]	6
hIL-5_{sc}-HSA + CHAPS	1.58 ± 0.11	N.D. [b]	3
HSA + CHAPS	1.36 ± 0.08	N.D. [b]	3
hIL-5_{sc}-HSA + cytosol/ATP	2.6 ± 0.15	1.26 ± 0.16	2
HSA + cytosol/ATP	1.4± 0.16	0.65 ± 0.22	2
hIL-5_{sc}-HSA + anti-Imp β antibody	2.2 ± 0.12	0.65 ± 0.28	2

[a] Results represent the mean ± S.E. (n indicated) from data (see Figs. 3.18, 3.19) from distinct experiments fitted to the function $Fn/c = Fn/c_{max}(1 - e^{-kt})$, where Fn/c_{max} is the maximal level of accumulation at steady state in the nucleus, k is the rate constant, and t is time in min. $t_{1/2}$ represents the time to reach half maximal accumulation.
[b] N.D. - not able to be determined.

To test whether this accumulation was due to an active transport process, the nuclear envelope was permeabilised using the detergent CHAPS (Efthymiadis *et al.,* 1997). Under these conditions, nuclear accumulation of hIL-5_{sc}-HSA was drastically reduced, nuclear levels being only 1.6-fold higher than in the cytoplasm, compared to 1.4-fold for HSA (Fig. 3.19, Panel C; Table 3.3). The conventional NLS-containing T-ag-NLS-β-Gal fusion protein showed comparable levels (Fn/c 1.2; Table 3.1). The results implied that hIL-5_{sc}-HSA was unable to bind to nuclear components, implying that nuclear accumulation of hIL-5_{sc}-HSA in the presence of an intact nuclear membrane as seen in Fig 3.15 (Panel A) occurs through an active transport process.







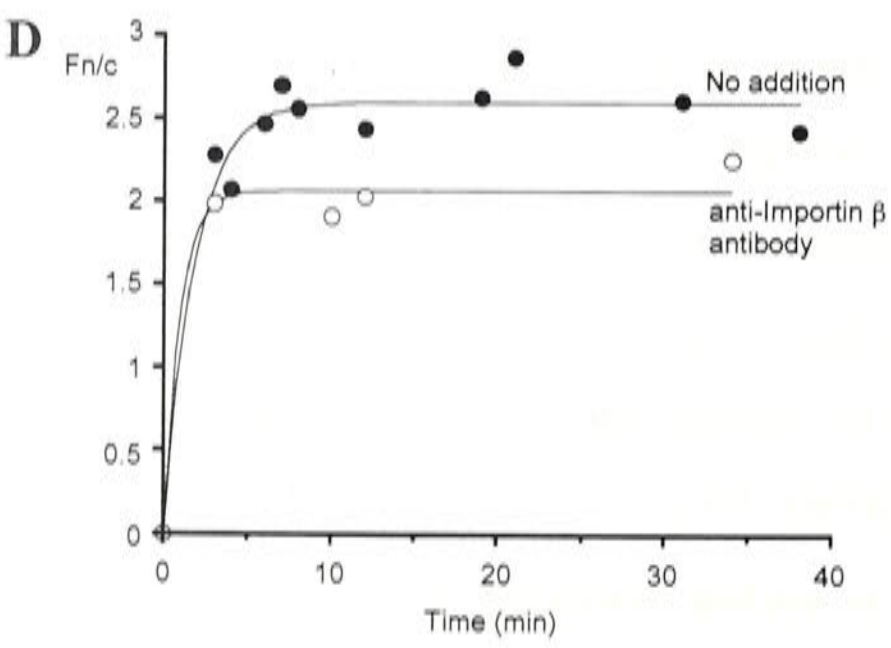


Fig. 3.19. Nuclear import of IL-5~HSA *in vitro*.
Nuclear import was reconstituted in mechanically perforated HTC cells. (A) CLSM images of nuclear accumulation of IL-5-HSA~Sfx after 30 min in the absence or presence of cytosol, CHAPS or anti-importin β antibody (40 μg/ml) as indicated. (B-D) Nuclear import kinetics from a single typical experiment performed as in (A) for IL-5-HSA~Sfx in the absence or presence of cytosol (B), for IL-5-HSA-Sfx or HSA~Sfx in the presence of CHAPS (C) or for IL-5-HSA~Sfx in the presence of anti-importin β antibody (D) as indicated. Each data point represents at least three separate measurements for each of Fn (nuclear fluorescence), Fc (cytoplasmic fluorescence), and background fluorescence. Data were fitted for the function $Fn/c = Fn/c_{max}(1-e^{-kt})$, where Fn/c_{max} is the maximal level of nuclear accumulation, k is the rate constant, and t is time in minutes. Pooled data are presented in Table 3.3.

3.13 Anti-importin β antibody does not prevent nuclear accumulation of IL-5_{sc}-HSA

The observation that hIL-5_{sc}-HSA can translocate into the nucleus of mechanically perforated HTC cells in the absence of cytosolic factors suggests that IL-5_{sc}-HSA import is independent of conventional importin and Ran-dependent pathways. However, cytosolic factors may not be completely depleted after cell permeabilization, with a small pool of importin β known to remain associated with the NPC (Görlich *et al.,* 1995; Fagotto *et al.,* 1998; Schedlich *et al.,* 2000), able to mediate nuclear import of factors such as insulin growth factor binding protein (IGFBP) 3. To exclude the involvement of importin β in the nuclear accumulation of hIL-5_{sc}-HSA, a specific antibody (Hübner *et al.,* 1999) was used in the *in vitro* nuclear transport assay as described in Section 2.9 of Materials and Methods.

Nuclear accumulation was not significantly reduced in the presence of anti-importin β antibody (Fn/c 2.2; Fig. 3.19, Panel D; Table 3.3), implying that importin β appears not to be involved in nuclear translocation of the IL-5_{sc}-HSA fusion protein.

The same anti-importin β antibody was active in inhibiting nuclear accumulation of the importin α/β-recognised NLS-containing T-ag-NLS-β-galactosidase fusion protein (see Table 3.1) as well as that of the importin β-recognised transcription factor CREB (Hübner *et al.,* 1991; Forwood, 2001) and IGFBP-3 (Schedlich *et al.,* 2000).

3.14 IL-5 is not recognized by the conventional NLS-binding importin α/β heterodimer

Whilst nuclear transport of the hIL-5wt-NLS-β-Gal fusion protein is dependent on cytosolic factors, the baculovirus-expressed IL-5 forms as well as the hIL-5_{sc}-HSA fusion protein did not require exogenous factors to translocate into the nucleus, implying that importins do not play a role in their nuclear localization. A previously established enzyme-linked

immunosorbent assay (ELISA)-based importin binding assay was used (Efthymiadis *et al.*, 1997) to investigate whether importin α/β recognizes the IL-5 NLS. IL-5-NLS-β-Gal fusion proteins were coated onto microtiter plates, incubated with increasing amounts of importin α-GST, importin β-GST or pre-dimerized importin α/β-GST complex, and binding was quantitated using antibodies specific to GST and an alkaline phosphatase-labelled secondary antibody as described in Section 2.10 of Materials and Methods. In comparable fashion to previous measurements (Efthymiadis *et al.*, 1998), the positive control T-ag-NLS β-Gal fusion protein was recognized by the importin α/β-GST complex with high affinity, showing an apparent dissociation constant (k_D) of 10 nM (Fig. 3.20).

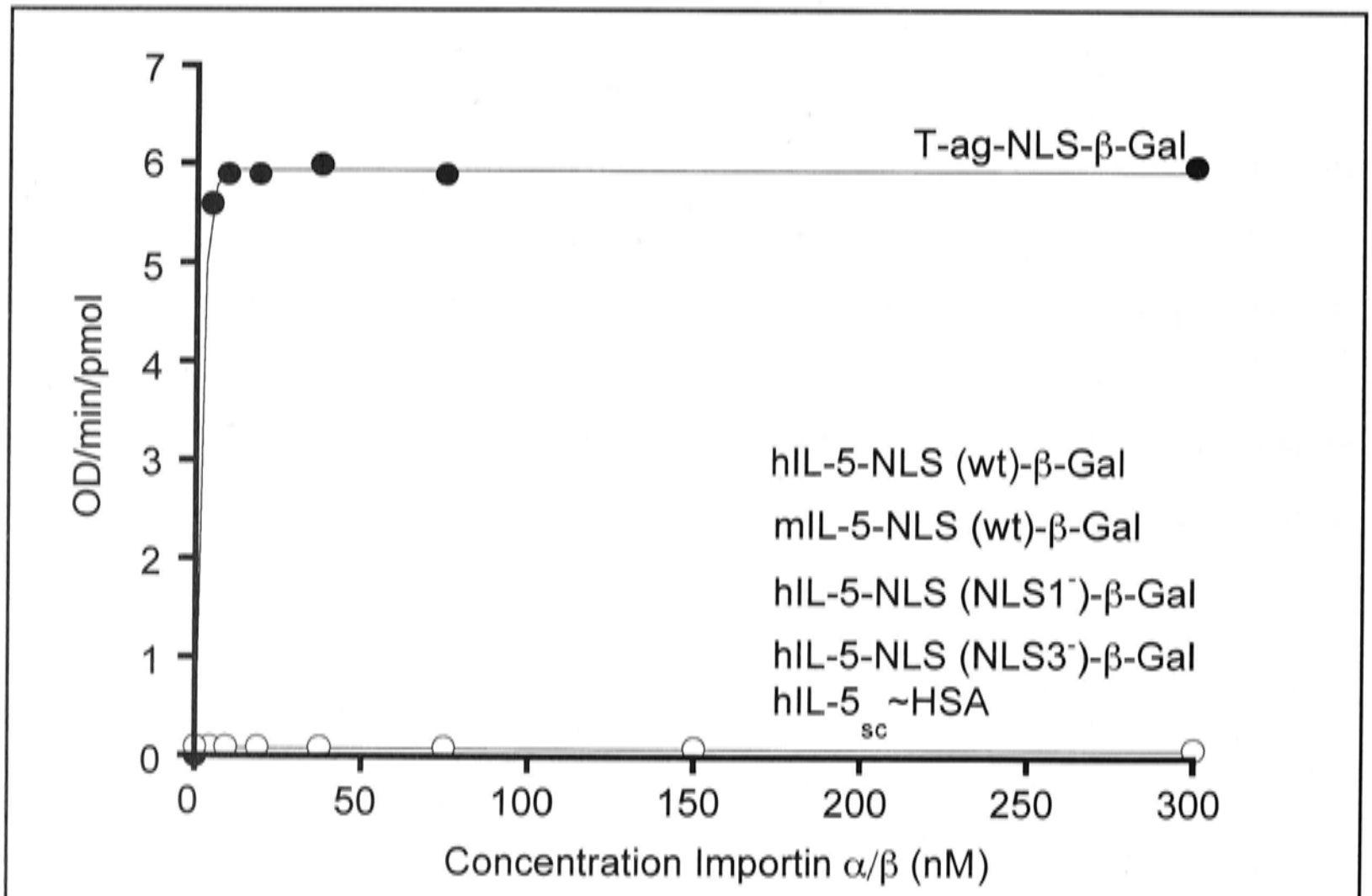


Fig. 3.20. The NLS regions of mIL-5wt, hIL-5wt and mutant derivatives and hIL-5sc~HSA are not recognized by importins as quantitated using an ELISA-based binding assay. T-ag-NLS-β-Gal, mIL-5wt-NLS-β-Gal and mutant derivatives were expressed in *E.coli* as described in section 2.4. of Material and Methods. hIL-5-HSA was expressed in baculovirus system. Microtiter plates were coated with either T-Ag-NLS-β-Gal, mIL-5wt-NLS-β-Gal, hIL-5wt-NLS-β-Gal, or hIL-5wt-β-Gal and derivatives and incubated with increasing concentrations of importin subunits. Data were fitted for the function $B(x) = B_{max} (1 - e^{-kx})$, where x is the concentration of importin, and B the level of importin bound, with the apparent dissociation constants indicated.

In contrast, the mouse and human wildtype IL-5-NLS-β-Gal fusion proteins as well as their NLS mutant derivatives exhibited no significant binding of the importin α/β-GST complex above that of β-galactosidase alone (Fig. 3.20).

To exclude the possibility that this result was a consequence of the fact that the IL-5-NLS region is out of context in the tetrameric β-Gal fusion protein, even though it is functional in terms of NLS activity, an ELISA was used to investigate whether importin α/β would recognize the IL-5_{sc}-HSA fusion protein. IL-5_{sc}-HSA showed no detectable recognition of the importin α/β-GST complex above that of HSA alone (Fig. 3.20). This lack of binding of the IL-5_{sc}-HSA fusion protein by the conventional importin α/β NLS receptor was consistent with the *in vitro* nuclear transport results in that nuclear accumulation of the IL-5_{sc}-HSA fusion protein does not require cytosolic factors.

3.15 Discussion

In this chapter, the mechanism of nuclear transport of IL-5 was investigated using an established *in vitro* nuclear import assay, representing the first quantitative analysis of nuclear transport of several mouse and human IL-5 NLS mutant derivatives and a human

IL-5_{sc}-HSA fusion protein. Initial experiments (Jans *et al.*, 1997a) had implied that human IL-5 contained a conventional bipartite NLS, but other studies (Calanni, 1997) and those presented here indicate that NLS3 rather than NLS2 (see Fig. 3.7) is a critical component of the functional NLS of hIL-5. Whilst the hIL-5wt-NLS was able to cotarget β-galactosidase into the nucleus, the hIL-5 $NLS3^{-}$ mutant fusion protein was completely excluded, implying an involvement of this basic region in nuclear transport of IL-5. It therefore seems reasonable to conclude that the hIL-5 NLS is not a classical bipartite NLS, but rather comprises a single cluster of lysine residues - **KK** YIDGQ **KKK**^{104}, which is not recognized by the conventional importin α/β heterodimer. This NLS, however, appears to be functional in targeting β-galactosidase to the nucleus in an *in vitro* nuclear transport system. Ostensibly, nuclear import of this fusion protein seems to be through a conventional nuclear import pathway, in that it is dependent on ATP and cytosolic factors, and is inhibited by the non-hydrolysable GTP analogue GTPγS. Since the hIL-5-NLS-β-Gal protein is not recognized by importin α/β, however, it would

seem that it does not enter the nucleus through a conventional importin α/β-mediated pathway. Additionally, the NLS region of mIL-5, in contrast to the hIL-5 NLS, was not able to target β-galactosidase to the nucleus, despite the presence of cytosolic factors and ATP. Clearly, under *in vitro* conditions the NLS of mouse IL-5 (**KK YIDRQ KEK**[103]) is not sufficient for nuclear targeting of such a large heterologous protein.

In contrast to hIL-5-NLS-β-Gal, hIL-5, mIL-5 as well as hIL-5-HSA are transported into the nucleus without a requirement for exogenously added ATP or cytosolic factors. Additionally, nuclear transport of IL-5 was not blocked by GTPγS, indicating that Ran may not play a role in this process. Even though the size of baculovirus-expressed IL-5 is below the molecular weight cut-off of the nuclear pore, it is clear from the CHAPS experiments described here that nuclear accumulation of hIL-5 and mIL-5 observed *in vitro* is achieved by active transport, rather than passive diffusion and subsequent binding to nuclear components. Further, since IL-5's *in vivo* role (see Chapter 5) may be to cotransport receptor molecules to the nucleus, examination of its nuclear import in isolation may not be fully comparable to the *in vivo* nuclear transport of a large IL-5 receptor complex.

Clearly, a significant difference was observed in the nuclear import characteristics of the β-Gal fusion protein compared to the IL-5-HSA fusion construct. This discrepancy may be explained by the distinct features of the molecules used. First of all, the IL-5-β-Gal protein only contained the NLS region of IL-5 and forms a large, tetrameric complex, whilst the HSA protein was fused to the complete IL-5 sequence, consisting of a single-chained dimer.

In accordance with the above findings, different experimental approaches suggest that IL-5 in its baculovirus-expressed form, or as a β-galactosidase or HSA fusion protein, is not recognized by the conventional importin α/β proteins, indicating that these do not mediate IL-5-NLS-dependent nuclear import. The possibility cannot be excluded that other importins or indeed other novel proteins factors are responsible for nuclear translocation of IL-5, since it has been observed that importins are not always completely removed during the perforation process in the *in vitro* nuclear transport assay (Schedlich *et al.*, 2000; Forwood *et al.*, 2001). Whatever the case in this regard, it seems clear that importin α/β is not involved.

Since the HSA fusion protein contains the entire IL-5 sequence, novel importins or factors others than those involved in the conventional nuclear import may potentially

recognize regions of IL-5 other than the proposed NLS cluster and contribute to nuclear transport in an ATP and Ran-independent fashion as shown for a variety of other nuclear proteins (see Sections 1.2.5 and 6.2).

The distinct requirements for *in vitro* nuclear transport of hIL-5wt-NLS-β-Gal and hIL-5 indicate that the β-galactosidase fusion protein is not an appropriate model to investigate IL-5 nuclear transport. This especially seems to be the case for mIL-5. Whilst mIL-5 nuclear transport is comparable to that of hIL-5 in all respects, mIL-5wt-NLS-β-Gal, in contrast to hIL-5wt-NLS-β-Gal, was completely excluded from the nucleus. Thus, the construction of a mIL-5-HSA fusion protein instead of mIL-5-NLS-β-Gal would allow further insight into the nature of the IL-5 NLS. Specifically, the features of the IL-5 NLS responsible for IL-5 nuclear translocation need to be further investigated. The HSA fusion protein would be most suitable as it contains the entire IL-5 molecule, shows comparable nuclear transport properties in terms of requirement for exogenously added cytosolic factors and ATP, and its size exceeds the molecular weight cut-off (50 kDa) of the NPC. Whether these requirements for the hIL-5sc-HSA nuclear import reflect those for a putative IL-5~IL-5 receptor complex remains to be determined.

IL-5 seems to be able to interact directly with the nuclear envelope, implied by inhibition experiments with WGA and importin β, which presumably is the basis of the lack of a requirement for interaction with importin α/β for nuclear import. The competition by importin β suggests that either common or closely adjacent docking sites at the NPC for IL-5 and importin β may be involved. Analysis of the ability of IL-5 to bind nucleoporins would clarify this question.

A problem of concern for future *in vivo* studies is the fact that amino acids within the putative NLS region of IL-5 are important for receptor binding, making mutagenic studies difficult. As shown here for the mIL-5 NLS1^{-} mutant derivative, NLS mutations can abolish high affinity binding to the IL-5 receptor, making conclusions about the physiological role of these NLS regions *in vivo* difficult. It is therefore an absolute requirement to verify receptor binding for NLS mutant derivatives, and the flow cytometry technique established here will undoubtedly be invaluable in such studies.

CHAPTER 4 SUBCELLULAR LOCALIZATION OF IL-5 *IN VIVO*

4.1 Introduction

That IL-5 has the ability to translocate into the nucleus has been documented using several different experimental systems, including *in vitro* (see Chapter 3) and *in vivo* (Jans *et al.*, 1997a) nuclear transport assays, and transfected hIL-5 receptor expressing FDC-P1 mouse myeloid progenitor cells (which express the β_c receptor endogenously), treated with fluorescently labelled human IL-5 (Jans *et al.*, 1997a). All of these systems revolve around the use of fluorescence microscopy, as do other indirect immunofluorescence studies, indicating that IL-5 can be targeted into the nucleus of an IL-5 receptor expressing human myeloid cell line AML14.3D10 (Calanni, 1997). This cell line can partially differentiate into eosinophils in the presence of IL-5 combined with GM-CSF (Paul *et al.*, 1993).

The aim of the work presented in this chapter was to use approaches other than fluorescence microscopy to confirm the ability of IL-5 to localise in the nucleus subsequent to receptor-mediated endocytosis (Jans *et al.*, 1997a), where IL-5, in contrast to the *in vitro* nuclear import assay described in the previous chapter, has to gain access to the nucleus by passing two consecutive, intact membrane systems, i.e., the cell membrane as well as the nuclear membrane of receptor-expressing cells.

Three different approaches were utilized in order to assess the subcellular localization of IL-5 in a recently derived FDC-P1 cell line (FDC-mIL-5R) expressing the mouse IL-5α receptor subunit from the high expression pEFBOS vector (pcDEF3mIL5Rα; see Table 2.5 in Materials and Methods).

Firstly, baculovirus-expressed IL-5 was fluorescently labelled and internalisation and subcellular localization of mIL-5-Sfx in IL-5 receptor expressing cells was investigated by means of confocal laser scan microscopy (CLSM) as previously done for hIL-5 (Jans *et al.*, 1997a). Secondly, an immunogold labelling approach was used in which thin sections of IL-5 receptor bearing cells were immunostained with IL-5 specific antibodies and subsequently analysed by electron microscopy (Post-Embedding Immunogold Electron Microscopy). Thirdly, a novel, biochemical approach was developed, in which IL-5 receptor-expressing cells were incubated with biotinylated

IL-5, then subjected to cell lysis and subcellular fractionation with subsequent protein precipitation and immunoblotting.

4.2 Direct immunofluorescence studies of nuclear import of IL-5 in FDC-mIL-5R

Previous studies had used CLSM to show that hIL-5 can localise in the nucleus of FDC-P1 cells expressing the human IL-5 α receptor in receptor-specific fashion (Jans *et al.*, 1997a). Comparable studies were attempted using mIl-5 and FDC-P1 cells expressing the mouse IL-5 receptor. Baculovirus-expressed mIL-5 was fluorescently labelled as described in Section 2.3.4 of Materials and Methods. The mIL-5-Sfx derivative retained biological activity as shown in a proliferation assay with the mouse myeloma progenitor FDC-mIL-5R cell line (Table 4.1).

Table. 4.1. Biological activities of mouse and human IL-5 and their derivatives.

Protein	Biological activity [a] in units/mg
mIL-5(wt)	$2.7x10^7$
mIL-5(wt)-Sfx	$2.2x10^7$
mIL-5(NLS1$^-$)	$6.7x10^5$
mIL-5(NLS1$^-$)-Sfx	$5x10^5$
mIL-5(mono)	$4.6x10^7$
mIL-5(mono)-Sfx	$1.6x10^7$
hIL-5(wt)	$1.6x10^7$
hIL-5(wt)-Sfx	$1.5x10^6$
hIL-5_{sc}-HSA	$1x10^6$
hIL-5_{sc}-HSA-Sfx	$5x10^5$

[a] Biological activities varied from batch to batch; representative samples were chosen, with labelled and unlabelled forms for any one derivative compared for the same batch. Activities were determined as described in Section 2.7.3 of Materials and Methods.

In Fig. 4.1, the specific fluorescence for membrane-bound and nuclearly localized mIL-5~Sfx is shown. The specific fluorescence was determined by performing image analysis of cells incubated with IL-5~Sfx only (total binding) and cells incubated in IL-5~Sfx as well as unlabelled IL-5 in 10-fold excess (non-specific binding) as described in Section 2.13 of Materials and Methods. The difference between total and

non-specific fluorescence gave a measure of the specific fluorescence due to binding of mIL-5 to the IL-5 receptor at 37°C. The 10-fold excess of unlabelled IL-5 was able to compete for binding, demonstrating that the process of binding was indeed receptor-mediated. The membrane binding of IL-5 was rapid and reached maximum levels within the first 10 min. Over the time course of 400 min, the level of membrane binding did not drop markedly, implying that a steady state equilibrium between internalisation of the ligand-receptor complex and receptor recycling movement was reached. Specific nuclear fluorescence was detectable approximately 30 min after the addition of IL-5~Sfx, reaching maximum levels at around 120 min, with approx. 20 % of the membrane bound amount of IL-5, the kinetics being comparable to the previous study with hIL-5 (Jans *et al.*, 1997a). At 4°C the level of specific fluorescence at the membrane after 90 min was comparable to that at 37°C, but no nuclear mIL-5 was detectable.

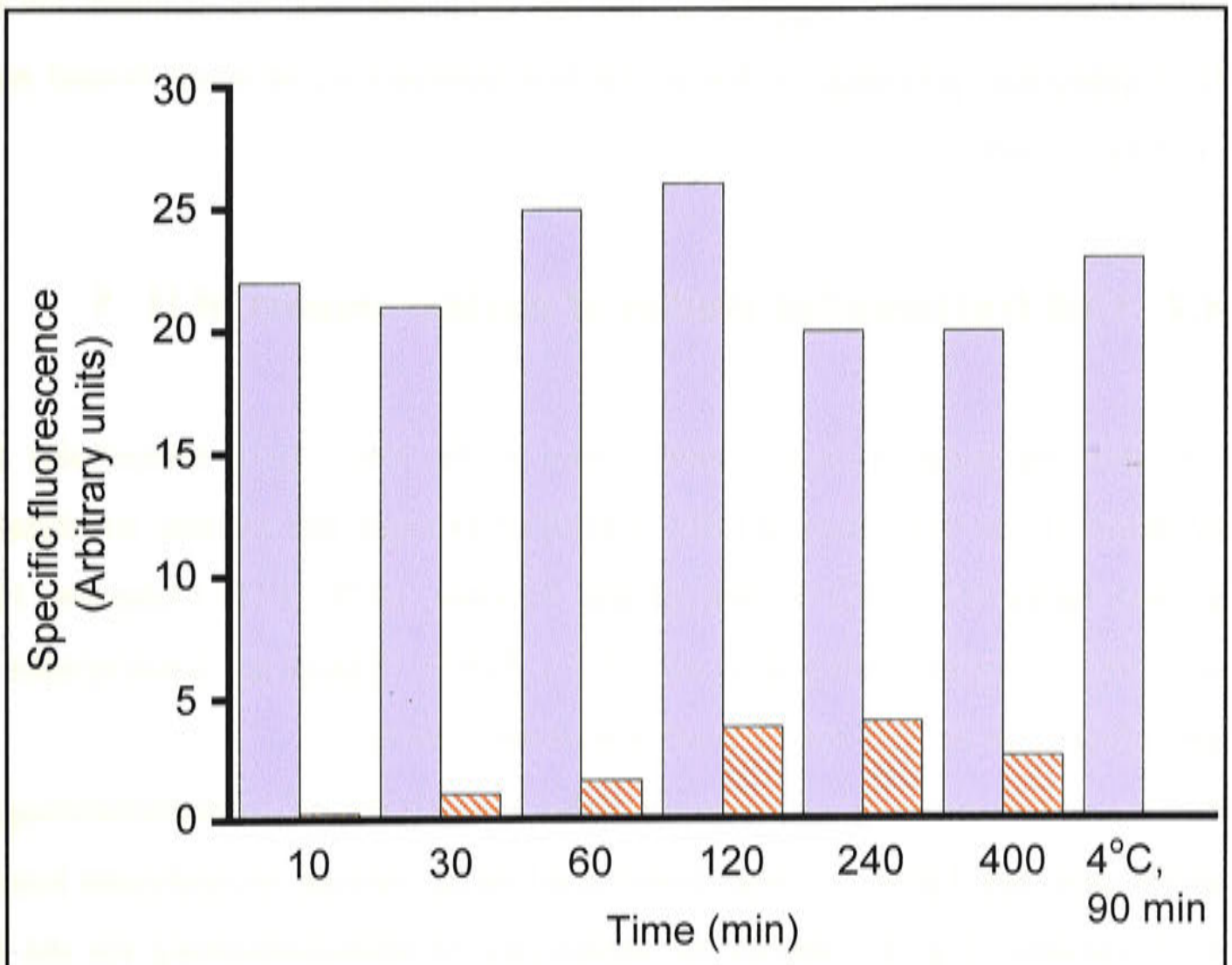


Fig. 4.1. Nuclear localization of mIL-5-Sfx *in vivo*.
Specific membrane and nuclear fluorescence due to membrane binding and internalisation of fluorescently labelled mIL-5 mouse by FDC-mIL-5R cells. Cells were incubated for various times at 37°C with 1 μM mIL-5(wt)~SFX in the absence and presence of a 10-fold excess of unlabelled mIL-5, prior to mounting and visualisation of fluorescence using CLSM as described in Section 2.13 of Materials and Methods. Image analysis was performed to quantitate membrane and nuclear fluorescence in the absence (total binding) and presence (non-specific binding) of unlabelled hIL-5, with the latter subtracted from the former to give a measure of the fluorescence due to specific binding (see Jans et al., 1997a).

The intrinsic difficulties of the quantitation procedure used in this approach, mainly due to the very high noise to signal ratio meant that alternative experimental approaches were necessary to verify the nuclear localization of IL-5 in intact cells.

4.3 Electron microscopic study of nuclear import of IL-5

The second approach to investigate nuclear localization of IL-5 *in vivo* used a post-embedding immunogold technique as described in Section 2.11 of Materials and Methods. Thin sections were prepared of cells cultured either with or without IL-5 as well as of cells either expressing or lacking the IL-5 α receptor. Sections were subsequently stained with primary anti-IL-5 antibody following incubation with 10 nm colloidal gold conjugated secondary antibodies. Analysis of these sections revealed no detectable signal at the membrane, in the cytoplasm or in the nucleus of FDC-mIL-5R cells cultured in IL-5, suggesting that the approach was not suitable for the purpose of IL-5 detection, presumably due to the low numbers of receptor-bound and internalised IL-5 molecules.

4.4 Cell fractionation studies of nuclear import of IL-5

Cellular fractionation is a powerful tool in the analysis of subcellular distribution of proteins. It has been successfully employed to show the nuclear localization of several growth factors like NGF (Yankner and Shooter, 1979), EGF (Johnson, 1980; Savion *et al.*, 1981), insulin (Goldfine *et al.*, 1977), PDGF (Rakowicz-Szulczynska *et al.*, 1986b) and others (for review see Burwen and Jones, 1987).

Prior to fractionation experiments, the sensitivity of immunoblotting of anti IL-5 antibodies (see Table 2.7) was determined using various recombinant human and mouse IL-5 variants. Fig. 4.2 shows the sensitivity of immunoblotting for the ligands tested, performed as described in Section 2.14 of Materials and Methods.

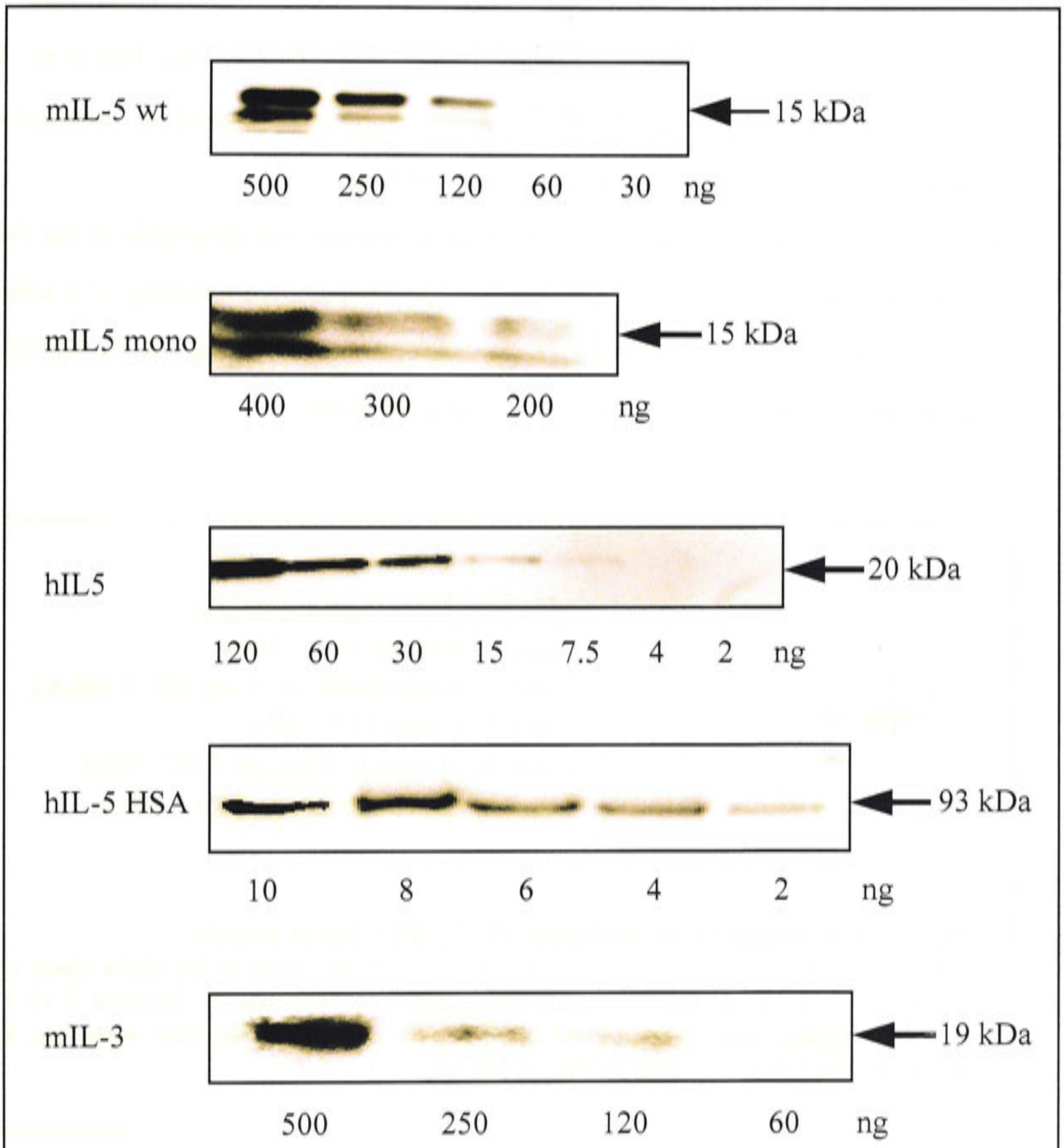
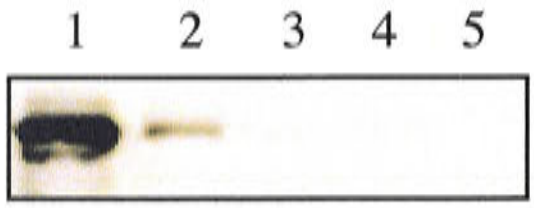


Fig. 4.2. Sensitivity of immunoblotting of recombinant mIL-5, hIL-5, mIL-3 and derivates thereof. Proteins were expressed using the baculovirus system in Sf9 cells and purified by gel filtration followed by ion-exchange chromatography. Samples were prepared for SDS-PAGE and immunoblotted with the respective antibodies (see Table 2.7) as described in Section 2.14 of Materials and Methods.

The detection limit for mouse IL-5 was very high. Specifically, 100 ng of mIL-5(wt) were needed for reliable detection, 200 ng of mIL-5(mono), and 100 ng of mIL-3. In contrast, immunoblotting of human IL-5 was 10- to 100-fold more sensitive, where as little as 10 ng of hIL-5(wt) and 1 ng of hIL-5_{sc}-HSA could be detected. Since the IL-5_{sc}-HSA fusion protein could be detected with the highest sensitivity, it was chosen for

subsequent cell fractionation experiments. The cells used were FDC-hIL-5R cells, which are stable transfectants of the hIL-5 receptor α chain in addition to the endogenous mIL-5 β-receptor subunit. $4x10^7$ FDC-hIL-5R cells (see Jans *et al.*, 1997a) were pre-incubated with 5 nM IL-5_{sc}-HSA overnight and prepared for immunoblotting as described in Section 2.15 of Materials and Methods.

Whilst IL-5_{sc}-HSA was detectable in the media, nothing was detectable in the cytosolic or nuclear fractions (Fig. 4.3). Consistent with this, immunoblotting of a whole cell lysate did not reveal any signal for IL-5_{sc}-HSA either, indicating that IL-5_{sc}-HSA was internalised in FDC-hIL-5R cells to only negligible levels.



hIL-5 sc HSA fusion protein
Lane 1: 400 ng hIL-5 HSA
Lane 2: supernatant (c. 4 ng hIL-5 HSA)
Lane 3: lysate FDC-hRα
Lane 4: cytosolic fraction FDC- hRα
Lane 5: nuclear fraction FDC- hRα

Fig. 4.3. Internalization of the human IL-5_{sc} HSA fusion protein.
FDC-P1cells stably transfected with the cDNA corresponding to the alpha chain of the human IL-5 receptor, were lysed or fractionated as described in Sections 2.15.1 and 2.15.2. Samples were prepared for SDS-PAGE and immunoblotted with anti hIL-5 antibody (see Table 2.7) as described in Section 2.14.

To investigate whether this lack of any detectable signal was due to interference with receptor-binding of the IL-5_{sc}-HSA fusion protein, the receptor binding properties of the IL-5_{sc}-HSA were examined using flow cytometry (see Section 2.12.1 of Materials and Methods). The receptor binding to the hIL-5_{sc}-HSA fusion protein was compared to that of hIL-5(wt) using FDC-hIL-5R cells. Cells were exposed to the fluorescently labelled hIL-5 in the range of 0.2-10 nM and analysed as described in Section 3.2.

Fig. 4.4 shows the total binding of hIL-5(wt)~Sfx to FDC-hIL-5R cells. Typical binding curves from the cytometric data were derived by plotting the relative fluorescence

versus the concentration of hIL-5(wt)~Sfx (Fig. 4.5). Panel A shows the total and non-specific binding of hIL-5(wt)~Sfx. The specific binding of hIL-5(wt)~Sfx to FDC-hIL5R cells is shown in panel B. The K_D value obtained from this typical binding curve of hIL-5(wt) to FDC-hIL-5R cells was 1.2 nM, which is of the same order of magnitude as published values (Ingley and Young, 1991).

In Fig. 4.6, the total binding of hIL-5_{sc}-HSA~Sfx to FDC-hIL-5R cells is shown. Typical binding curves were derived from the cytometric data by plotting the fluorescence versus the concentration of hIL-5_{sc}-HSA~Sfx (Fig. 4.7). The hIL-5_{sc}-HSA~Sfx fusion protein showed drastically reduced specific binding to FDC-hIL5R cells (Fig. 4.7 panel B), indicating that binding to the IL-5 receptor is of very low affinity. This result suggests that the lack of detection of this hIL-5 form in the immunoblotting experiments are likely to be caused by a deficiency in high affinity binding to the IL-5 receptor, rendering attempts to localize hIL-5_{sc}-HSA in the nucleus of FDC-mIL-5R cells impossible.

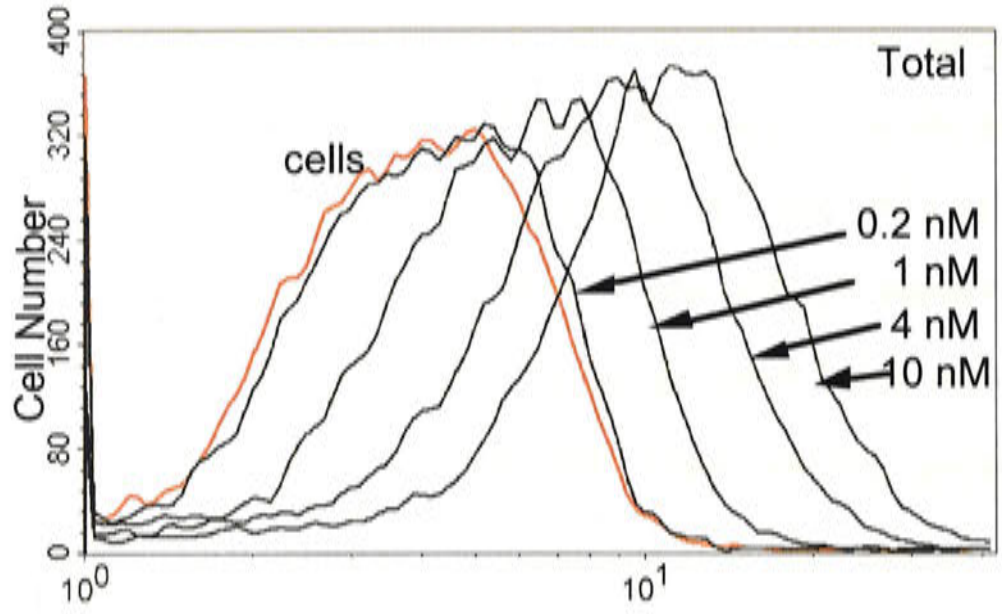


Fig. 4.4. Fluorescence histogram of FDC-hIL-5R cells incubated with increasing concentrations of human IL-5~Sfx.

FDC-P1 cells stably transfected with the cDNA for hIL-5Rα were incubated with increasing concentrations of hIL-5~Sfx at 4°C and subjected to flow cytometry as described in Section 2.12.1 of Materials and Methods.

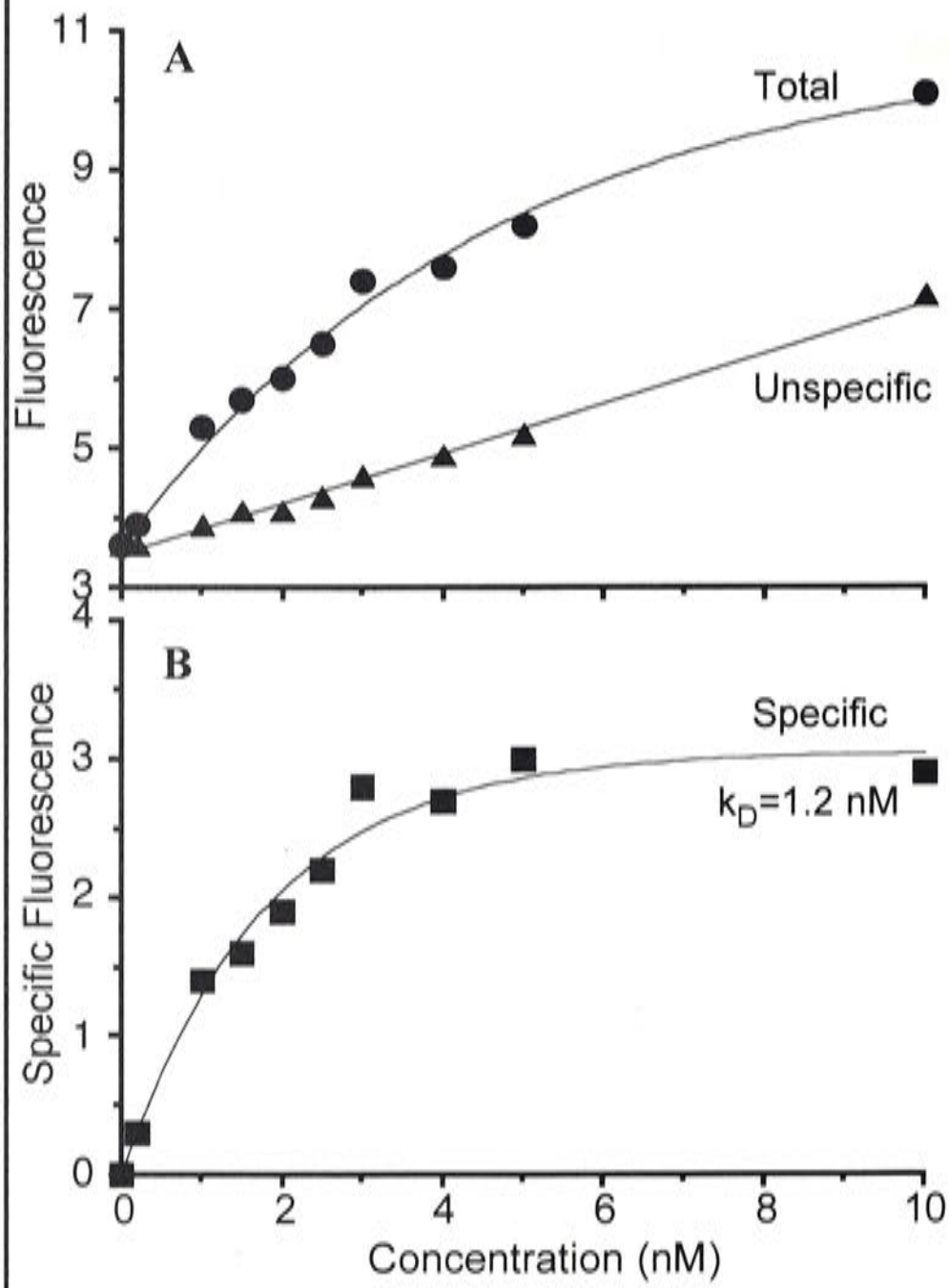


Fig. 4.5. Receptor binding of hIL-5~Sfx.

Typical receptor binding curves are shown. The data were obtained from cytometric profiles as in Fig. 4.4 and plotted as the geometrical mean of the fluorescence versus the concentration of hIL-5~Sfx.

The curve for total binding was obtained in the presence of fluorescently labelled ligand, while the non-specific curve was acquired in the presence of additional unlabelled ligand in 100-fold excess (Panel A).

The data were replotted for specific binding as the difference between the total and non-specific binding (Panel B).

The data were fitted for the function $B(x) = B_{max} (1 - e^{-kx})$, where B_{max} is maximal binding, k is the rate constant, and x is concentration of labelled hIL-5 in nM.

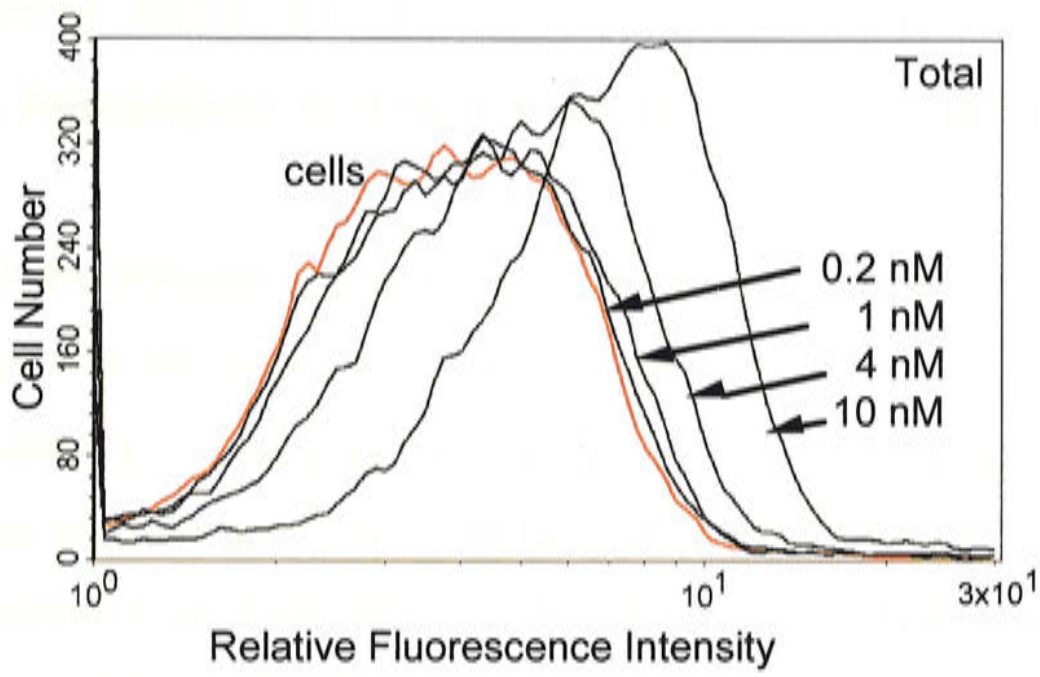


Fig. 4.6. Fluorescence histogram of FDC-hIL-5R cells, incubated with increasing concentrations of hIL-5_{sc}-HSA~Sfx.

FDC-P1 cells stably transfected with the cDNA for hIL-5Rα were incubated with increasing concentrations of hIL-5_{sc}-HSA~Sfx at 4°C and subjected to flow cytometry as described in Section 2.12.1 of Materials and Methods.

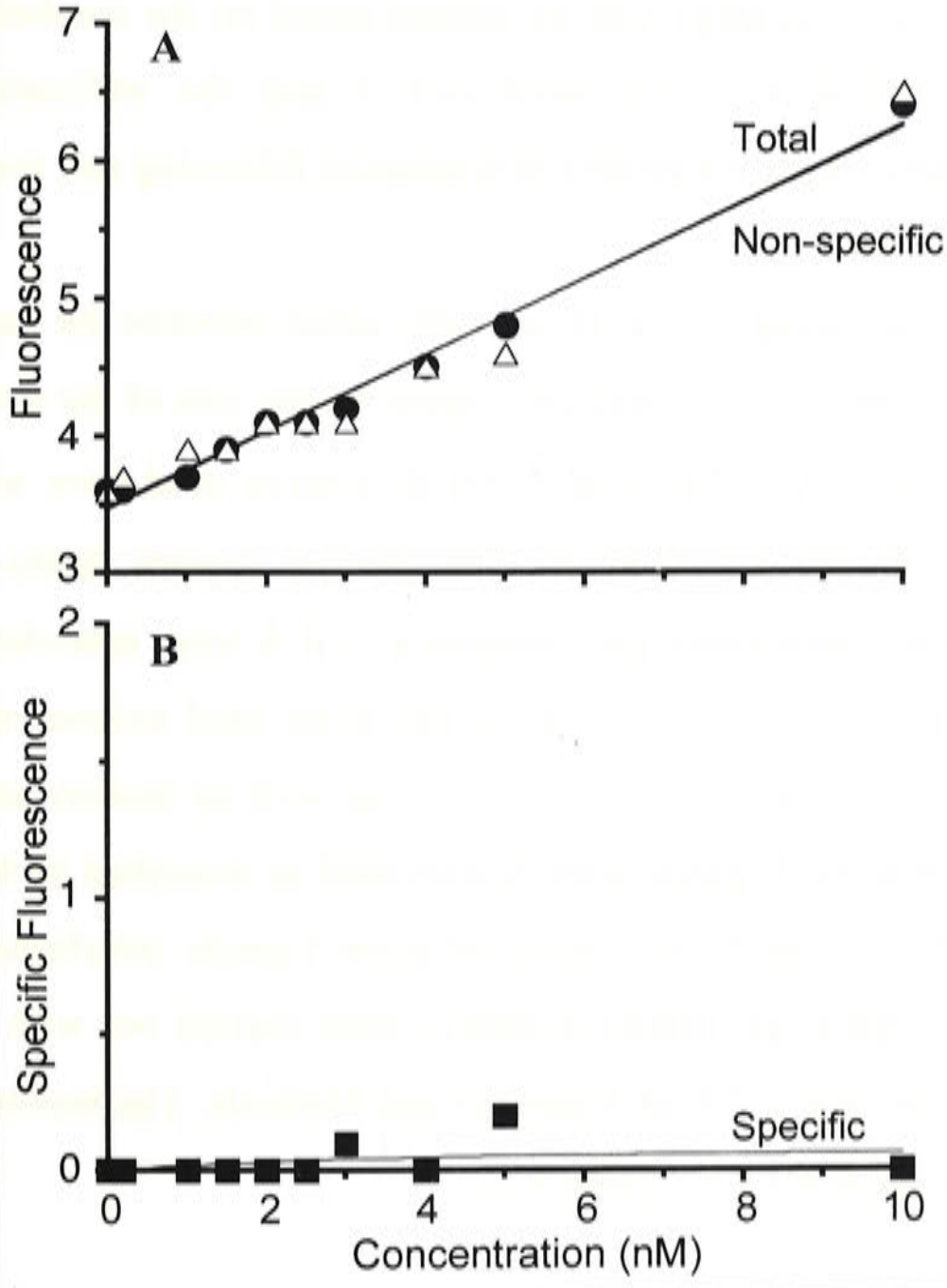


Fig. 4.7. Receptor binding of hIL-5_{sc}-HSA ~Sfx.

Typical receptor binding curves are shown. The data were obtained from cytometric profiles as in Fig. 4.6 and plotted as the geometrical mean of the relative fluorescence versus the concentration of hIL-5_{sc}-HSA~Sfx.

The curve for total binding was obtained in the presence of fluorescently labelled hIL-5_{sc}-HSA, while the non-specific curve was acquired in the presence of unlabelled ligand in 100-fold excess (Panel A).

The data were replotted for specific binding as the difference between the total and non-specific binding (Panel B).

The data were fitted for the function $B(x) = B_{max}(1 - e^{-kx})$, where B_{max} is maximum binding, k is the rate constant, and x is concentration of labelled ligand in nM.

4.5 Establishment of an immunoprecipitation procedure based on biotinylation of IL-5 and streptavidin-conjugated magnetobeads

As described in the Section 4.4, the sensitivity of immunoblotting of all recombinant ligands and their derivatives used was very low except for hIL-5_{sc}-HSA, which could not be used because of low receptor binding affinity. Another problem with the fractionation procedure was the fact that the large volumes of the nuclear fractions (500-1000 µl) made additional concentration steps such as Trichloroacetic acid (TCA) precipitation or use of centricons necessary, potentially leading to a loss of protein. This fact, together with the expected low amounts of IL-5 in the nucleus of IL-5 receptor-expressing cells subsequent to incubation at 37°C rendered successful detection of IL-5 in the nucleus of FDC-mIL-5R cells unlikely.

In order to overcome these problems and to circumvent the reliance on ligand-specific antibodies, an immunoprecipitation strategy was developed based on the incubation of IL-5 receptor-expressing cells with biotinylated mIL-5 and the utilization of streptavidin-conjugated magnetic beads for protein precipitation following cell lysis and fractionation of FDC-mIL-5R cells.

Biotinylation of proteins takes advantage of the strong association between the bacterial protein streptavidin from *Streptomyces spec.* and the vitamin biotin, one of the strongest non-covalent interactions known (10^{-15} M). The biotin derivative used here was the water-soluble Sulfo-NHS-LC-Biotin, which reacts with primary amines. Sulfo-NHS-LC-Biotin is unable to permeate membranes and contains a 22.4 Å long, non-cleavable spacer (Pierce, product information). This derivative has been used extensively for biotinylation of cell surface proteins (Altin *et al.*, 1995) as well as biotinylation of antibodies and recombinant proteins. Ligands were biotinylated as described in Section 2.3.5. To exclude the possibility that biotinylation of these ligands interfered with binding to their respective receptors proliferation assays were carried out with FDC-mIL-5R cells as described in Section 2.7.3. of Materials and Methods. The biotinylated derivatives retained their biological activity (Table 4.2).

Table 4.2. Biological activities of mIL-5 and mIL-3 and their biotinylated forms

Protein	Biological activity in units/mg
mIL-5(wt)	2.7×10^6
mIL-5(wt)~biotin	2.7×10^6
mIL-3(wt)	4.4×10^7
mIL-3(wt)~biotin	2×10^6

[a] Biological activities varied from batch to batch; representative samples were chosen, with labelled and unlabelled forms for any one derivative compared for the same batch. Activities were determined as described in Section 2.7.3 of Materials and Methods.

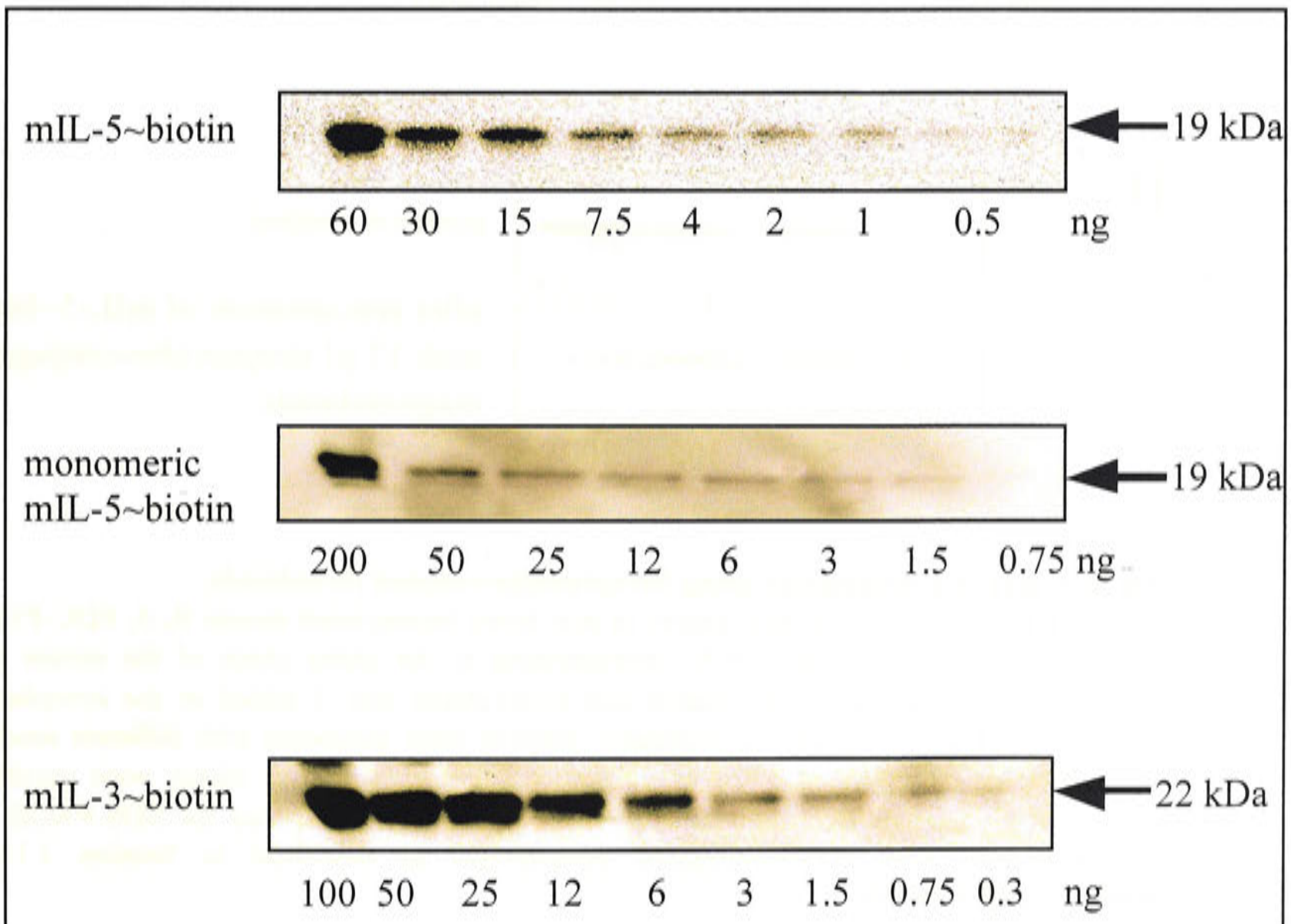


Fig. 4.8. Sensitivity of immunoblotting of biotinylated recombinant proteins mIL-5, monomeric mIL-5 and mIL-3.
mIL-5, mIL-5 (monomeric form) and mIL-3 were labelled with biotin, samples prepared for SDS-PAGE and immunoblotted with HRP-conjugated streptavidin as described in Section 2.14 of Materials and Methods.

In Fig. 4.8 the detection limits of various biotinylated ligands are shown. After biotinylation, the sensitivity of immunoblotting increased dramatically compared to immunoblotting using ligand-specific antibodies. Specifically, as little as 1 ng of mIL-5(wt)~biotin, 1.5 ng of mIL-5(mono)~biotin, and 0.75 ng of mIL-3~biotin could be detected.

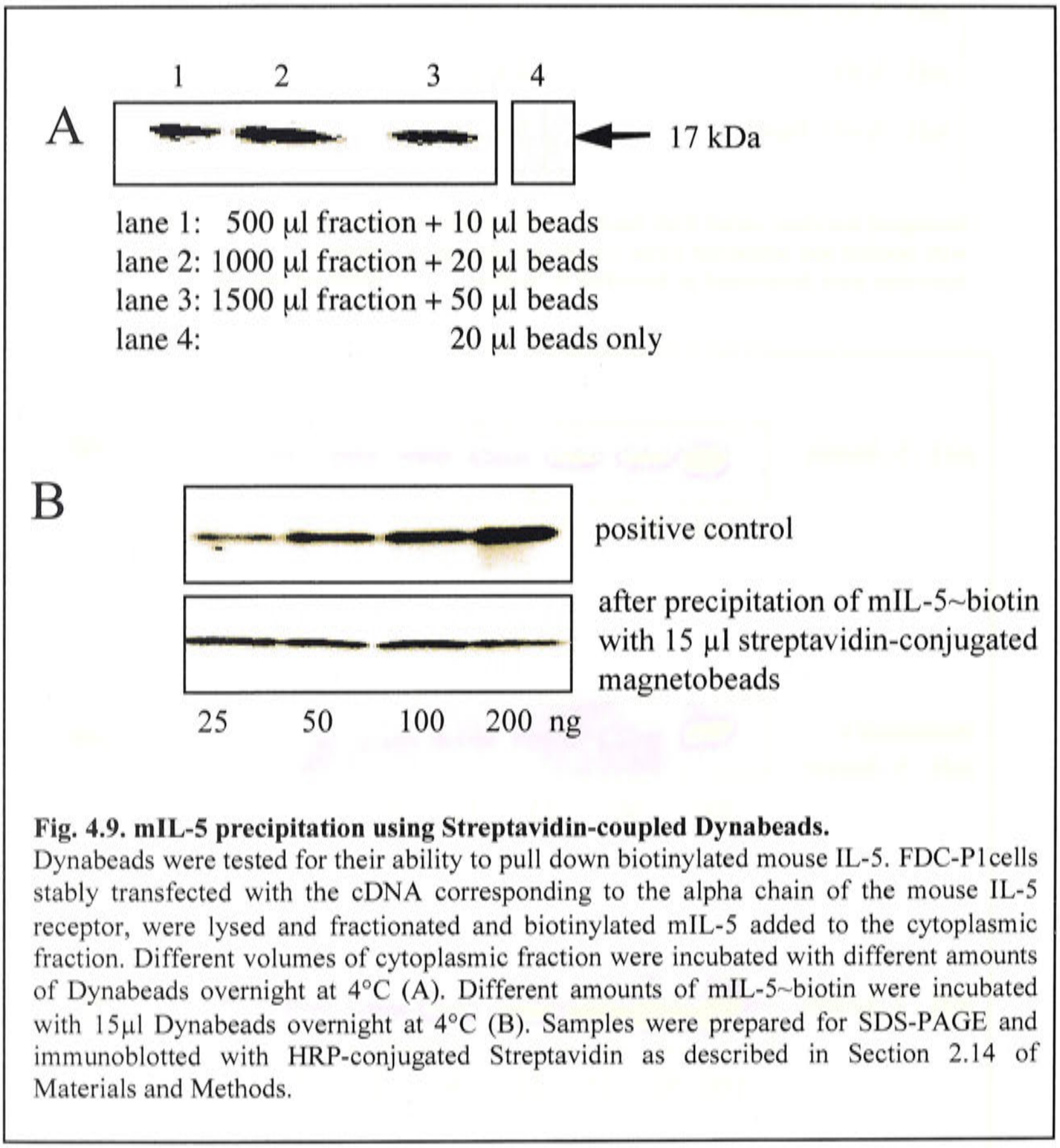


Fig. 4.9. mIL-5 precipitation using Streptavidin-coupled Dynabeads.
Dynabeads were tested for their ability to pull down biotinylated mouse IL-5. FDC-P1cells stably transfected with the cDNA corresponding to the alpha chain of the mouse IL-5 receptor, were lysed and fractionated and biotinylated mIL-5 added to the cytoplasmic fraction. Different volumes of cytoplasmic fraction were incubated with different amounts of Dynabeads overnight at 4°C (A). Different amounts of mIL-5~biotin were incubated with 15µl Dynabeads overnight at 4°C (B). Samples were prepared for SDS-PAGE and immunoblotted with HRP-conjugated Streptavidin as described in Section 2.14 of Materials and Methods.

The main advantage of ligand biotinylation is the possibility of precipitating IL-5 from large volumes using streptavidin-conjugated magnetic beads (Dynabeads, Dynal, Oslo, Sweden). To test the precipitation capabilities of this system, cytosolic fraction of FDC-mIL-5R cells pre-incubated with 5 nM mIL-5~biotin as described in section 2.15 of Materials and Methods were incubated with increasing amounts of beads. As shown in

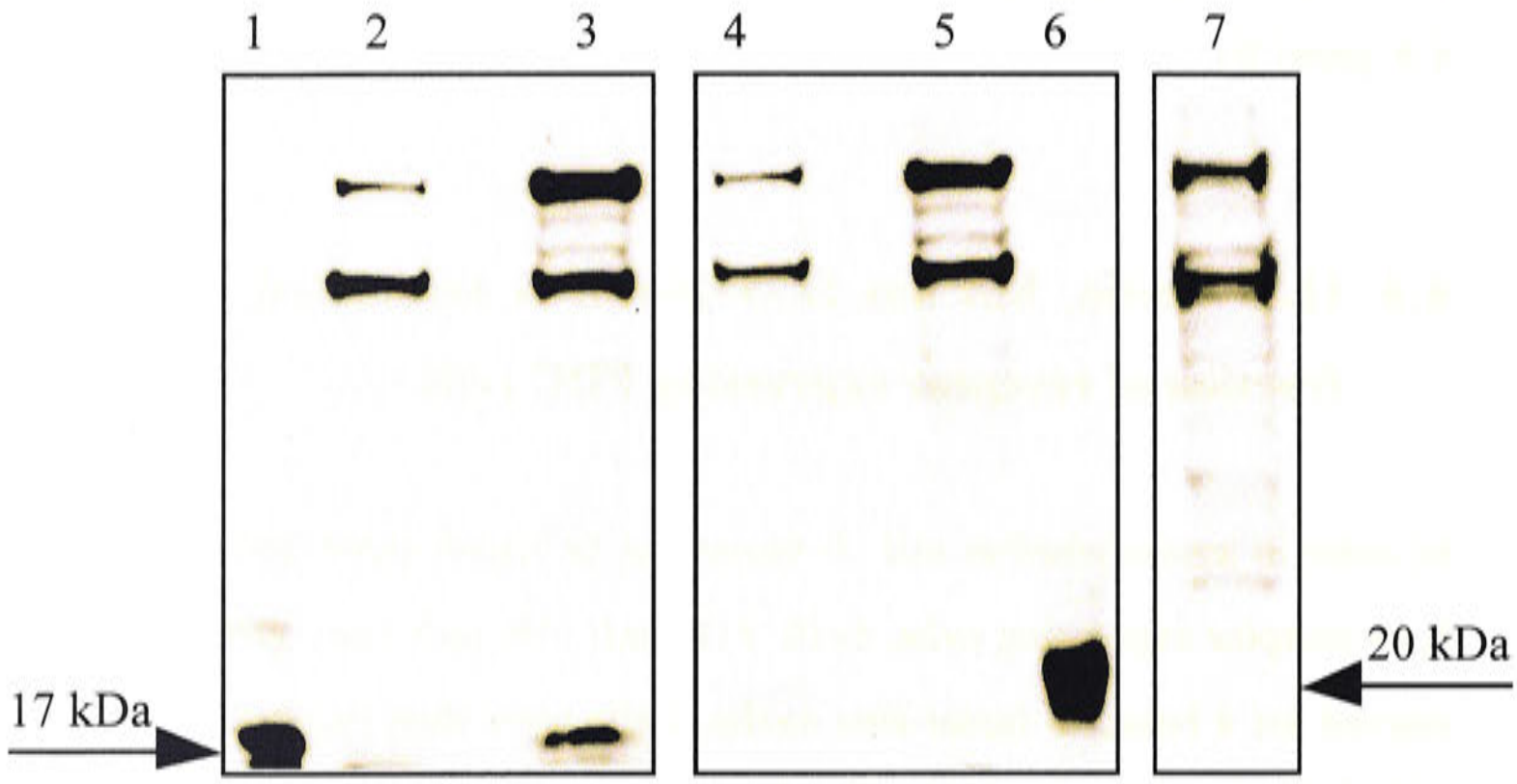
panel A (Fig 4.9), 10 µl of Dynabeads were sufficient to pull down biotinylated mIL-5 from 500 µl cytosolic fraction of FDC-mIL-5R. When 15 µl of beads were incubated with increasing amounts of IL-5~biotin, approximately 50 ng could be recovered (Fig. 4.9, panel B).

4.6 IL-5~biotin, but not IL-3~biotin is associated with the nuclear fraction of receptor expressing FDC cells

In order to assess whether mIL-5~biotin can be translocated into the nucleus of intact IL-5-receptor expressing cells, $4x10^7$ FDC-mIL-5R cells were grown and subsequently starved for 4 hours in factor-free media. Cells were then incubated overnight in 5 nM mIL-5~biotin under standard cell culture conditions and prepared for immunoblotting as described in Sections 2.14 and 2.15. Cell viability was verified before and after incubation with biotinylated factors as described in Section 2.12.2 of Materials and Methods. The percentage of dead cells was confirmed by flow cytometry, and only cell cultures with a vitality of 95 % or higher were used for further experimental procedures.

A typical immunoblot for nuclear fractions is shown in Fig. 4.10. mIL-5~biotin was associated only with the nuclear fraction of FDC-mIL-5R cells, expressing both IL-5 receptor subunits (Lane 3). In FDC-P1 cells lacking the α receptor subunit for mIL-5, no signal for mL-5~biotin could be detected (Lane 2). Likewise, when FDC-P1 cells, expressing both α and β subunits of the mIL-3 receptor endogenously, were incubated overnight in 5 nM mIL-3~biotin and prepared for immunoblotting, no signal for mIL-3~biotin could be detected in the nuclear fraction of these cells. In the negative control, where Dynabeads were incubated in the nuclear fraction of FDC-mIL-5R cells not pre-incubated with mIL-5~biotin, no signal was detected in the region of 15 to 25 kDa (lane 7), indicating that the signal for mIL-5 in the nuclear fraction of FDC-mIL-5R cells was specific for mIL-5~biotin.

These results suggest that mIL-5 is translocated to the nucleus in intact, IL-5-receptor-expressing cells. This is specific to IL-5, in that, even though the IL-3 receptor is expressed in the FDC-P1 cells, it does not occur for IL-3.



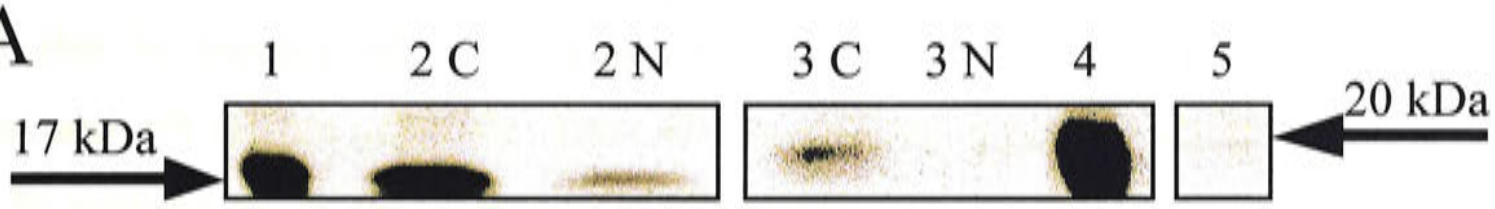
Lane	Cell type	Receptor for	Ligand	
1	positive control	-	mIL-5~biotin	15 ng
2	FDC-P1	mIL-3	mIL-5~biotin	
3	FDC-mIL-5Rα	mIL-3, mIL-5	mIL-5~biotin	
4	FDC-P1	mIL-3	mIL-3~biotin	
5	FDC-mIL-5Rα	mIL-3, mIL-5	mIL-3~biotin	
6	positive control	-	mIL-3~biotin	15 ng
7	negative control	-	beads alone	20 µl

Fig. 4.10. mIL-5, but not mIL-3, is associated with the nuclear fraction of myeloid progenitor cells FDC-mIL-5Rα.

FDC-P1 cells, stably transfected with the cDNA corresponding to the alpha chain of the mouse IL-5 receptor, were incubated with biotinylated mIL-5 or mIL-3 overnight. Nuclear fractions were prepared and subsequently precipitated with Streptavidin-conjugated Dynabeads overnight at 4°C. Samples were prepared for SDS-PAGE and immunoblotted with HRP-conjugated Streptavidin as described in Section 2.14 of Materials and Methods.

In order to estimate the relative amount of IL-5 localized in the nuclear fraction in comparison to the cytosolic fraction, both fractions were directly compared by immunoblotting.

A



Lane	Cell type	Fraction	Ligand	
1	positive control	-	mIL-5~biotin	15ng
2 C	FDC-mIL-5Rα	cytosol	mIL-5~biotin	
2 N	FDC-mIL-5Rα	nuclear	mIL-5~biotin	
3 C	FDC-P1	cytosol	mIL-3~biotin	
3 N	FDC-P1	nuclear	mIL-3~biotin	
4	positive control	-	mIL-3~biotin	15ng
5	negative control	-	beads alone	20µl

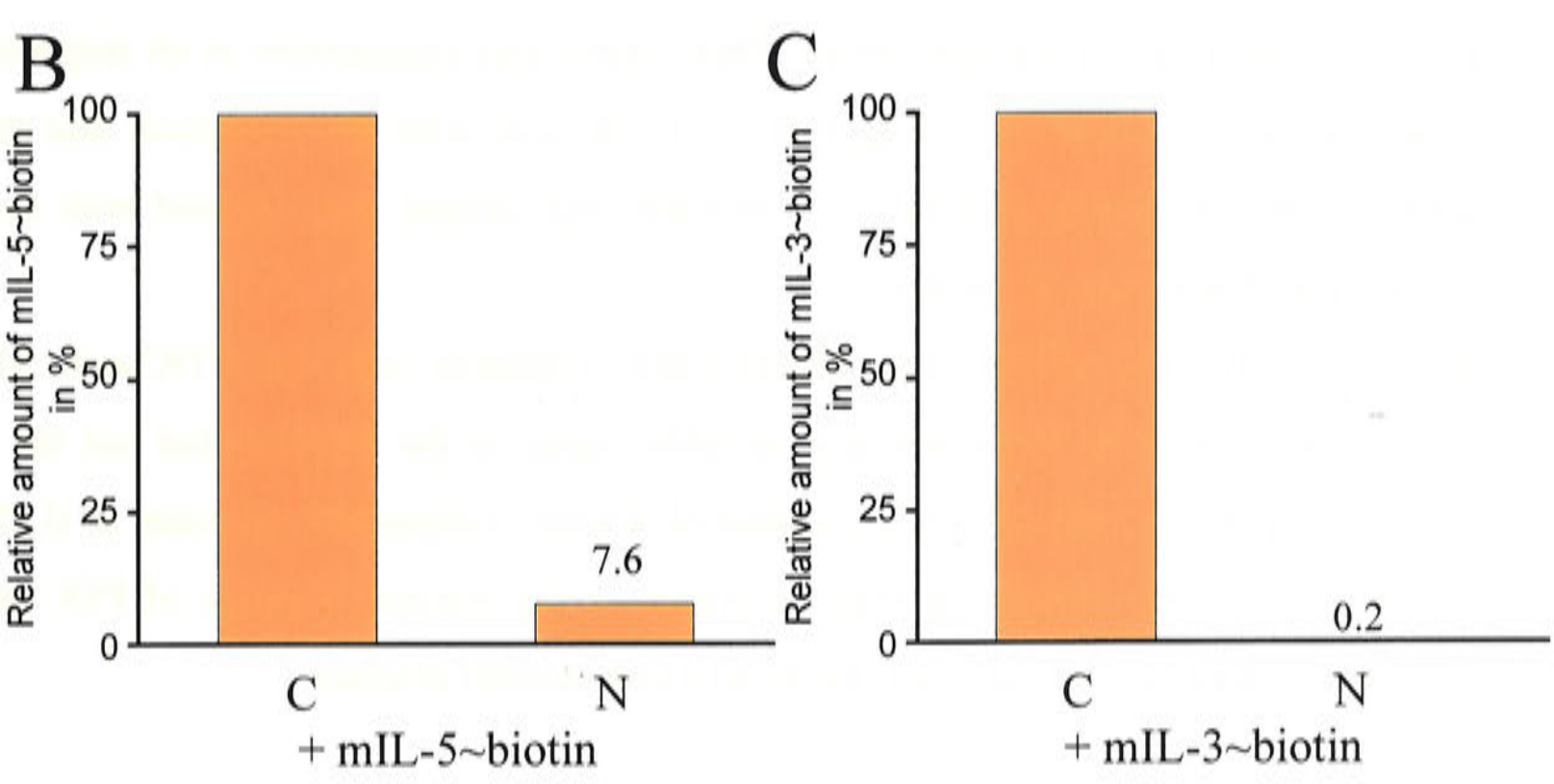


Fig. 4.11. The cytosolic and nuclear distribution of mIL-5~biotin and mIL-3~biotin in FDC-P1/mIL-5Rα cells.

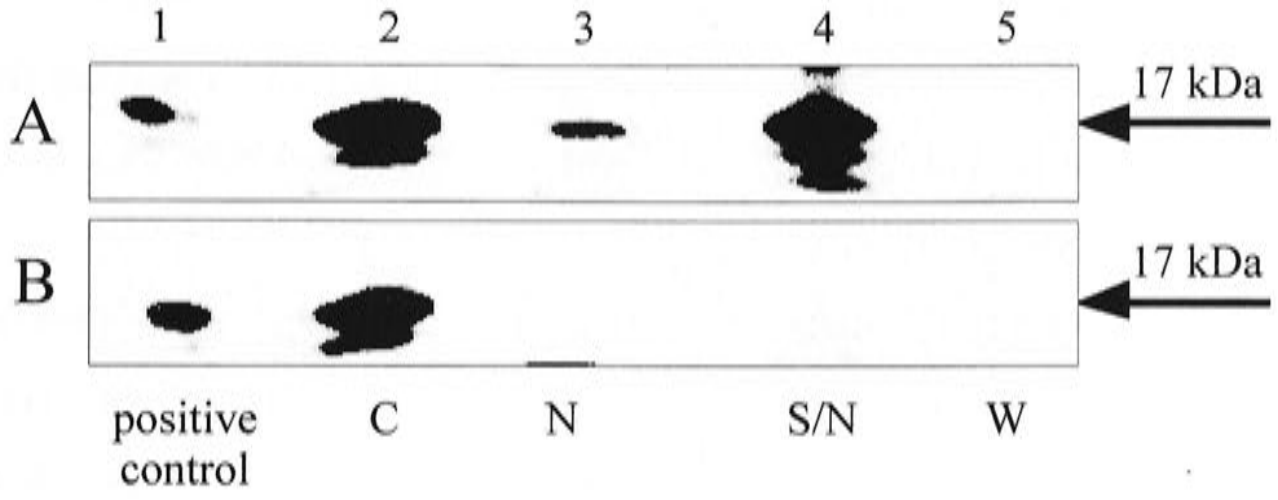
FDC-P1 cells, stably transfected with the cDNA corresponding to the alpha chain of the mouse IL-5 receptor, were incubated with biotinylated mIL-5 or mIL-3 over night. Cytoplasmic (C) and nuclear (N) fractions were prepared and subsequently precipitated with Streptavidin-conjugated Dynabeads overnight at 4°C. Samples were prepared for SDS-PAGE and immunoblotted with HRP-conjugated Streptavidin (A). Protein levels in the cytoplasm (B) and in the nucleus (C) were quantitated as described in Section 2.14.3 of Materials and Methods.

Panel A (Fig. 4.11) shows that mIL-5~biotin could be detected in the cytosolic fraction as well as in the nuclear fraction of FDC-mIL-5Rcells.

In contrast, mIL-3~biotin was associated exclusively with the cytosolic fraction, and could not be detected in the nuclear portion of mIL-3 receptor-expressing cells. The immunoblots were subjected to quantitative analysis using Image Gauge software (Fuji, Japan) as described in Section 2.14.3. Compared to the amount of mIL-5~biotin associated with the cytosolic fraction of FDC-mIL-5R cells, almost 8% was present in the nucleus. In comparison, only a faint signal was detected in the nucleus of FDC-P1 cells incubated in mIL-3~biotin (Fig. 4.11 A and B). These results indicated that only a small fraction of IL-5 was translocated to the nucleus of IL-5 receptor expressing cells, the greater part of receptor-bound ligand presumably remaining at the membrane or traffics through the lysosomal pathway. The low cytosolic amount of IL-3 complicates a comparison with IL-5 since the numbers of expressed cell surface receptors for IL-3 is greatly reduced compared to that for IL-5.

To exclude the possibility that the mIL-5~biotin signal detected in the nucleus of FDC-mIL-5R cells was caused by contamination during the lysis and fractionation procedure, a control fractionation was performed. This control was comparable in all respects to the experimental procedure involving FDC-mIL-5R cells with the exception that the cells were not pre-incubated with mIL-5 overnight, but instead were treated with cell lysis buffer spiked with mIL-5~biotin.

When immunoblots of both procedures were compared, only the FDC-mIL-5R cells pre-incubated with IL-5 showed a detectable signal in the nucleus, but not the spiked control (Fig. 4.12). This difference appeared despite comparable amounts of IL-5 in the cytosolic fractions, indicating that the signal in the nuclear fraction of FDC-mIL-5R cells was indeed specific and not due to an experimental artefact.



Lane 1: 15 ng mIL-5~biotin
Lane 2: cytoplasmic fraction FDC-mIL-5Rα (C)
Lane 3: nuclear fraction FDC-mIL-5Rα (N)
Lane 4: supernatant cell culture (S/N)
Lane 5: wash between C and N (W)

Fig. 4.12. Control for cytoplasmic contamination of the nuclear fraction.

FDC-P1 cells, stably transfected with the cDNA corresponding to the alpha chain of the mouse IL-5 receptor, were incubated with mIL-5~biotin (A) or nonbiotinylated mIL-5 (B) overnight. Cytoplasmic (C) and nuclear (N) fractions were prepared as described in Section 2.15. To verify that the nuclear fraction was not contaminated with cytoplasmic proteins during the fractionation procedure, a control (B), comparable in all respects but not incubated with mIL-5~biotin was spiked with biotinylated mIL-5 immediately prior to lysis for fractionation. Subsequently mIL-5~biotin was precipitated by incubation of all fractions with Streptavidin-conjugated Dynabeads overnight at 4°C. Samples were prepared for SDS-PAGE and immunoblotted with HRP-conjugated Streptavidin as described in Section 2.14.

4.7 Discussion

This chapter reports the first biochemical evidence that IL-5 can be translocated into the nucleus of an IL-5 receptor expressing cell line subsequent to receptor-mediated endocytosis. Cell fractionation experiments using a biotinylated form of mIL-5 showed that nuclear translocation is specific for IL-5, with the control experiments in Fig. 4.12 demonstrating that the results are not attributable to cross-contamination of cytosolic and nuclear fractions.

The biochemical approach developed here required the use of sufficiently high cell numbers to ensure that the number of IL-5 molecules per sample was higher than the

detection limit. Subsequent SDS PAGE and immunoblotting permitted the discrimination between background noise and specific signal for IL-5.

Importantly, biotinylation of the ligands had the advantage of making their detection in the cell fractions independent of ligand-specific antibodies, facilitating direct comparison between the IL-3 and IL-5 ligands.

By comparison, the electron microscopy approach was unsuccessful, presumably because the small amount of native antigen on the surface of the sections is beyond reliable detection. This result is consistent with previously published immunogold staining of eosinophils (Dubucquoi *et al.*, 1994) and mast cells (Wilson *et al.*, 2000), where no IL-5 was detected in the nucleus of those cells.

Direct immunofluorescence studies, in contrast, did detect an increase over time of specific signal in the nucleus of cells exposed to IL-5, but had the problem of background signal accounting for much of the overall signal, which made validation difficult (see however Jans *et al.*, 1997). Additionally, the amount of IL-5 used in the biochemical approach presumably is with 5 nM much closer to physiological concentrations than the amount used in the immunofluorescence studies (c. 1 μM).

Further investigations should of course include primary cells like eosinophils to clarify whether nuclear transport of IL-5 can also occur in primary target cells under physiological conditions.

CHAPTER 5 SUBCELLULAR LOCALIZATION OF THE IL-5 RECEPTOR IN INTACT CELLS

5.1 Introduction

Over the past three decades much evidence has been accumulated to support the hypothesis that membrane receptors of growth factors and cytokines can also be localized in the nucleus, implying that these receptors may have a direct role in intracellular and nuclear signalling in addition to their well-established role of mediating signal transduction at the cell membrane. Well-studied examples for nuclearly localized receptors are those for NGF (Rakowicz-Szulczynska, 1986), EGF (Rakowicz-Szulczynska, 1989), FGF-1 (Prudovsky, 1994), FGF-2 (Maher, 1996), IL-1 (Curtis, 1990) and GH (Lobie, 1994; Waters, 1994).

In previous investigations using an *in vitro* nuclear transport assay, mIL-5 appeared to be able of cotargeting the extracellular domains of the IL-5 receptor as a receptor complex to the nucleus of rat hepatoma HTC cells (Jans *et al.*, 1997ab). Additionally, indirect immunofluorescence studies with intact, IL-5 receptor expressing cells also indicated nuclear localization of the full-length, membrane bound IL-5 receptor α and β subunit (Calanni, 1997). Immunofluorescence results, however, need to be treated carefully, especially when the background to signal ratio is very high. Confirmation of observations using biochemical or other approaches is clearly desirable.

Cell fractionation has the advantage of permitting the separation of proteins by size before immunoblotting. Additionally, immunofluorescence studies with antibodies specific to one particular region of the antigen do not answer the question of whether the protein is present in its complete or partially degraded form.

The aim of this chapter was to provide evidence for the nuclear localization of the IL-5 receptor in intact cells by using subcellular fractionation, and to assess whether the receptor is co-localized in the nucleus with IL-5. To this end, biochemical analysis was performed, in which IL-5 receptor-expressing cells were subjected to cell lysis and fractionation with subsequent immunoblotting procedure.

5.2 Immunoblotting of the receptor subunits of the IL-3/IL-5/GM-CSF receptor system using subunit-specific antibodies

To establish whether nuclear localization is specific for the IL-5 receptor system and does not occur for the IL-3 or GM-CSF specific α receptor subunits, commercially available antibodies specifically recognizing the IL3, IL-5 or GM-CSF receptor α chains were tested for their ability to detect their antigens.

Mouse myeloid progenitor FDC-P1 cells, expressing the receptor systems for all three ligands were cultured in the presence of one of the three baculovirus-expressed IL-3, IL-5 or GM-CSF for 4 weeks and then subjected to a whole cell lysis procedure as described in Section 2.15.1. Lysates were then prepared for SDS Page and immunoblotting with the respective primary antibodies (see Table 2.7) and the appropriate horseradish-peroxidase (HRP)-conjugated secondary antibodies (see Table 2.8), prior to analysis using the LAS-1000 system and Image Gauge software.

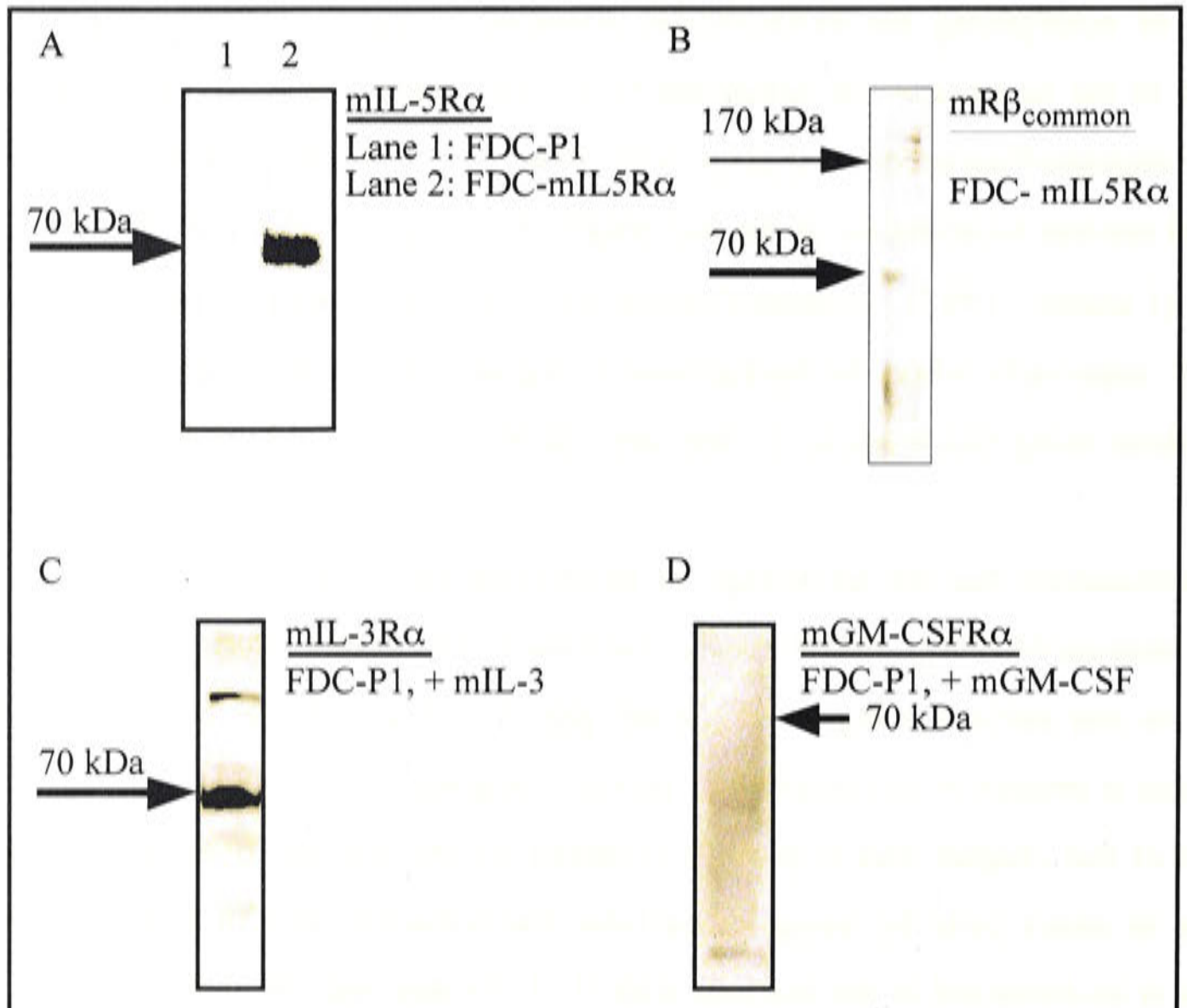


Fig. 5.1. Immunoblotting of receptor subunits for mouse IL-5, IL-3 and GM-CSF. Cells of the myeloid progenitor cell line FDC-P1, stably transfected with the mouse IL-5 receptor α subunit, were lysed as described in Section 2.15.1. Samples were prepared for SDS-PAGE and immunoblotted with the respective antibodies (see Table 2.7).

As shown in Fig. 5.1, a single specific, approx. 70 kDa band was detected for the mIL-5 receptor α subunit of FDC-mIL-5R cells, which did not appear in the lysate of FDC-P1 cells lacking the IL-5 receptor α chain (panel A). No signal could be detected for the $m\beta_c$ subunit (panel B). For mIL-3 receptor α, one specific band was visible at c. 70 kDa, and one additional band appeared above 100 kDa (panel C). No signal was detectable for the mGM-CSF receptor α chain (panel D). The lack of signal for β_c and GM-CSF Rα chain presumably is due to the low quality of the commercial antibody.

5.3 Subcellular localization of the mIL-5 receptor α chain in mIL-5 receptor expressing FDC cells

The evaluation of the capability of the receptor-specific antibodies to recognize their respective receptor subunits indicated that immunoblotting using anti-IL-5 receptor α antibody was sufficiently sensitive to detect the IL-5R α subunit in whole cell lysates.

In order to assess the subcellular localization of the mIL-5 receptor, FDC-P1 cells as well as FDC-mIL-5R cells were grown in IL-3 or IL-5 respectively, prior to subcellular fractionation (Section 2.15).

Cells with a vitality of 95 % or higher were used for further experimental procedures, and before nuclear lysis, the integrity of the nuclei was confirmed by light microscopy. To avoid contamination of the nuclear fraction with cytosolic components, nuclei were washed twice before nuclear lysis (Section 2.15.2).

As shown in Fig. 5.2, the mIL-5 receptor α subunit could be detected in both the cytosolic as well as in the nuclear fraction of FDC-mIL-5R cells cultured in mIL-5 (lanes 3 and 4). The immunoblotting revealed that the endocytosed mIL-5 receptor α associated with the nuclear fraction was intact, and no breakdown products were detected on the immunoblot, indicating that the receptor reached the nucleus in its full-length form. In contrast, no receptor was detected in the fractions of FDC-P1 cells lacking the mIL-5 receptor α subunit, indicating the specificity of the result.

A

Lane	Cell type	Cell fraction	Ligand	Cells/lane
1	FDC-P1	cytosol	mIL-3	10^6
2	FDC-P1	nuclear	mIL-3	2.5×10^7
3	FDC-mIL-5Rα	cytosol	mIL-5	10^6
4	FDC-mIL-5Rα	nuclear	mIL-5	2.5×10^7
5	FDC-mIL-5Rα	cytosol	mIL-3	10^6
6	FDC-mIL-5Rα	nuclear	mIL-3	2.5×10^7

B

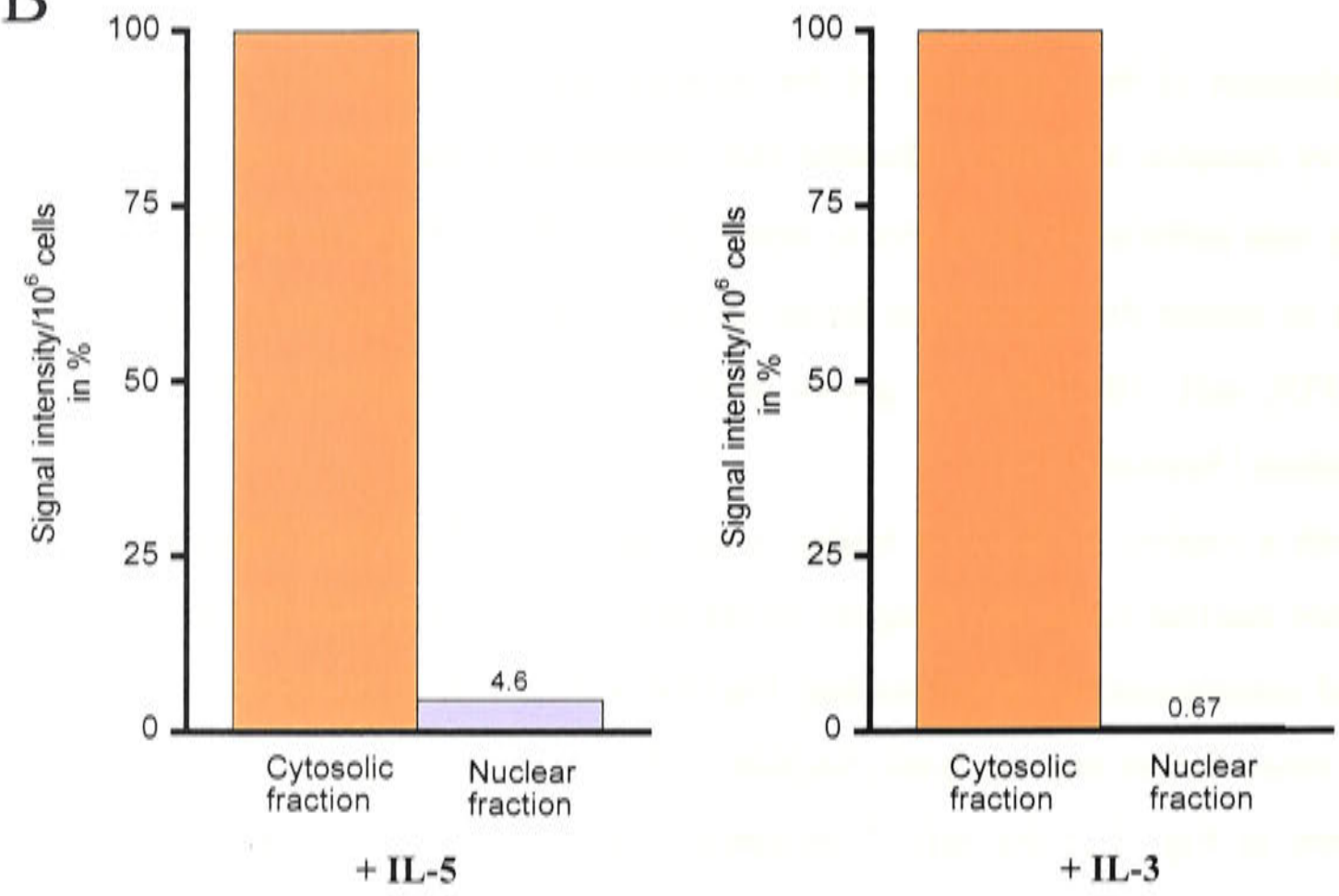


Fig. 5.2. Nuclear localization of the mouse IL-5 receptor alpha subunit is dependent on mIL-5.

Cytosolic and nuclear extracts were prepared from FDC-mIL-5Rα cells (see Section 2.15.2). Extracts were prepared for SDS-PAGE, and immunoblotted with anti-IL-5Rα antibody (A). Protein levels in the cytoplasm and in the nucleus (B) were quantitated by densitometry (ECL Plus, Amersham) using Image Gauge software, and values normalized to amount protein/10^6 cells.

5.4 Nuclear localization of mIL-5 receptor α is dependent on IL-5

If the *in vitro* observations regarding IL-5 cotargeting its receptor subunits to the nucleus (Jans *et al.*, 1997b; Calanni, 1997) are physiologically relevant, than nuclear localization of the IL-5 receptor would be predicted to occur in an IL-5-dependent fashion in intact cells expressing the full length, membrane bound receptor. To investigate the IL-5 dependence of the receptor nuclear transport, FDC-mIL-5Rα cells were cultured without mIL-5 for a period of four weeks in the presence of mIL-3 and subcellular localization of the IL-5 receptor compared to FDC-mIL-5Rα cells constantly cultured in mIL-5.

Subsequently cells were subjected to subcellular fractionation prior to SDS-PAGE and immunoblotting with an IL-5 receptor α specific antibody (see Sections 2.14 and 2.15). Equal amounts of proteins were loaded for the cytosolic fractions (corresponding to approx. 1×10^6 cells) as well as nuclear fractions (corresponding to approx. 2.5×10^7 cells). While the amount of IL-5 receptor α subunit in the cytoplasmic fractions of cells growing with and without IL-5 were similar, the IL-5Rα signals in the two nuclear fractions differed significantly (Fig 5.2, lanes 4 and 6 respectively). Quantitative analysis using the Image Gauge software (Fuji, Japan) indicated that the nuclear fraction of FDC-mIL-5R cells growing in medium depleted of IL-5 only showed approx. 15 % signal compared to that of cells grown continuously in IL-5.

To assess the time frame in which this change takes place, a time course experiment was performed, where FDC-mIL-5R cells were grown in either the presence or absence of IL-5 from 5 min up to 4 weeks. Following cell harvest and wash steps, cells were lysed and fractionated as described in Section 2.14. Samples of cytosolic fraction (approx. 1×10^6 cells) and nuclear fraction (approx. 1×10^7 cells) were prepared for SDS-PAGE and immunoblotted with anti mIL-5Rα antibody.

In agreement with Fig. 5.2, the amount of cytosolic IL-5 receptor α remained constant over the entire period of the experiment (Fig. 5.3, panel A-**C**), which was expected since the receptor was constitutively expressed in FDC-mIL-5R cells. The analysis of the immunoblots proved that the signal difference for IL-5 receptor α in the cytosolic fractions at all time points was no bigger than 6 %, indicating that the depletion of IL-5

in the medium over a longer period had no significant influence on the level of mIL-5Rα expression and therefore on the proportion of cells stably expressing the IL-5 receptor α in the entire cell population.

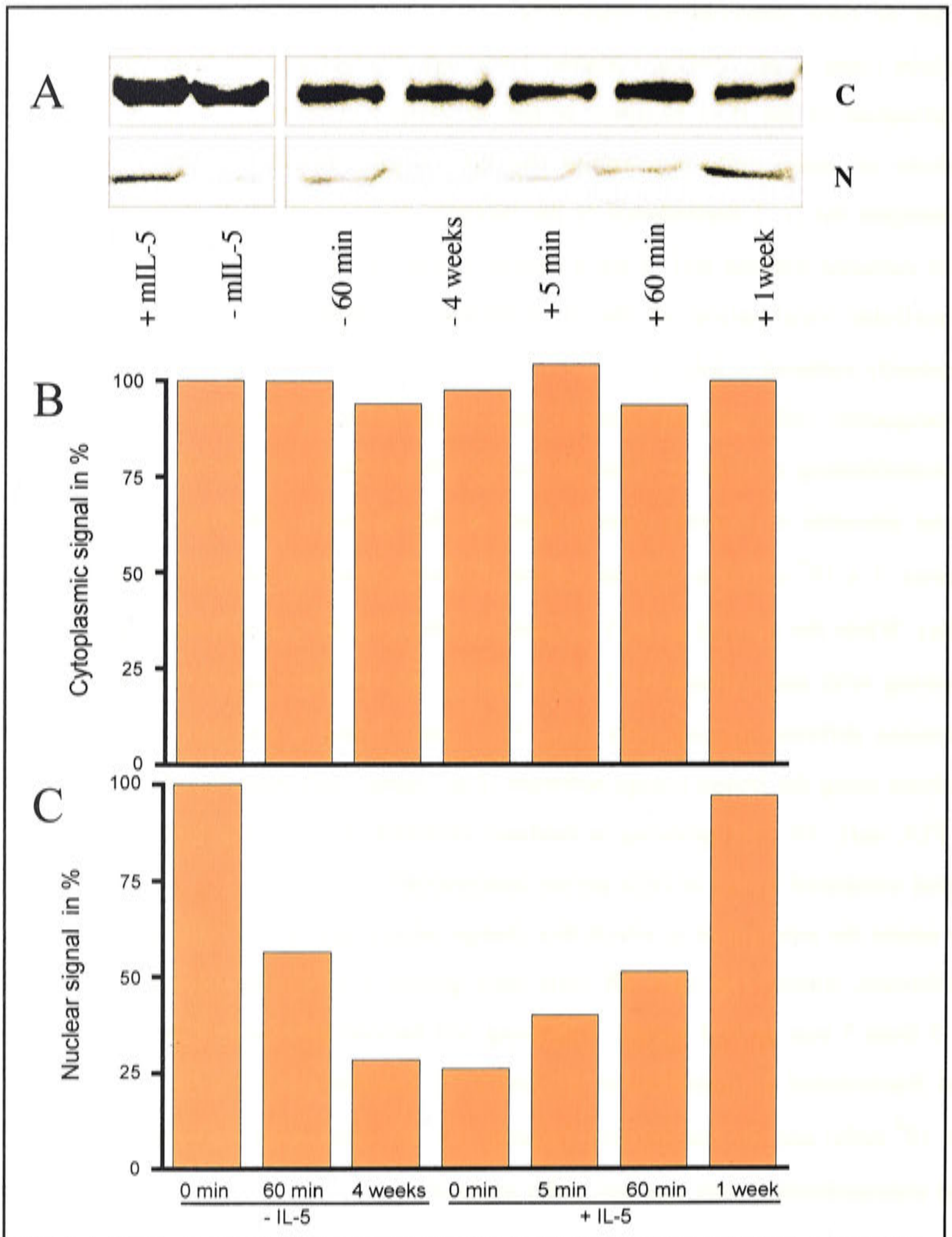


Fig. 5.3. Time course of IL-5 induced nuclear localization of the mouse IL-5 receptor α subunit.

FDC-mIL-5Rα cells were incubated with baculovirus-expressed mIL-5 for various amounts of time. Cytosolic and nuclear extracts were prepared for SDS-PAGE and immunoblotting with anti-IL-5Rα antibody (A). Protein levels in the cytoplasm (B) and in the nucleus (C) were quantitated by densitometry (ECL Plus, Amersham). Values were normalized to the respective cytosolic and nuclear values for cells cultured constantly in mIL-5.

In contrast, the nuclear fractions showed marked differences in the levels of IL-5Rα signals (Fig. 5.3, panel A-**N**). Analysis of the immunoblots of the nuclear fractions showed that with increasing time of IL-5 depletion the amount of nuclear IL-5 α receptor is reduced to about 25 % after 4 weeks (see Fig. 5.3). Specifically, the decline is most drastic within the first 60 min (to approx 50 %), with the lowest point of nuclear IL-5 receptor α after about 4 weeks (Fig. 5.3, panel C). This reduction in nuclearly localized IL-5 receptor α could be reverted by adding IL-5 to the cell culture, but only over the course of one week the original level was reached, implying that the nuclear accumulation of the mIL-5 receptor α subunit is directly dependent on the presence of IL-5.

5.5 mIL-5 receptor α can be co-precipitated with mIL-5~biotin from the cytosolic fraction of FDC-mIL-5R cells

The experimental data thus far had indicated that both IL-5 (Chapter 4) and the IL-5 receptor α subunit (this Chapter) are associated with the cytosolic and the nuclear fractions of FDC-mIL-5R cells. The experimental data were obtained independently for IL-5 as well as for its receptor α chain in the presence of IL-5. The question remains as to whether IL-5 and the IL-5 receptor α subunit remain associated within the nucleus, assuming that the two are cotransported to the nucleus as has been shown *in vitro* (Jans *et al.*, 1997b; Calanni, 1997).

Co-immunoprecipitation experiments were performed utilizing a biotinylated derivative of baculovirus-expressed mIL-5 (Chapter 4). To this end, FDC-mIL-5R cells were incubated overnight with 5 nM IL-5~biotin prior lysis and subcellular fractionation as described in Section 2.14. Cytosolic and nuclear fractions were subjected to protein precipitation using streptavidin-conjugated magnetobeads overnight, samples prepared for SDS-PAGE and subsequently immunoblotted. After staining for IL-5~biotin, the immunoblots were stripped and re-stained for detection of co-immunoprecipitated mIL-5 receptor α (see Section 2.14). While IL-5 was present in both cytosolic and nuclear fraction (Fig. 5.4), the IL-5 receptor α subunit was detected in the cytosolic fraction, but in the nuclear fraction only a faint band was observed, suggesting that IL-5 and its α receptor subunit may dissociate in the course of their nuclear import.

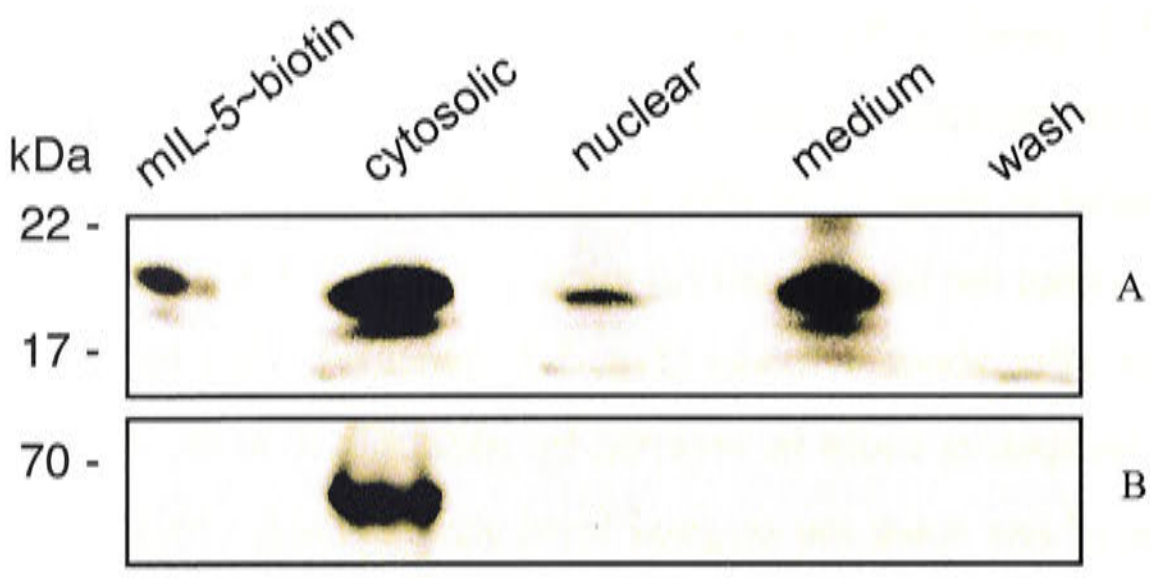


Fig. 5.4. Co-immunoprecipitation of the mIL-5 Receptor α subunit.
FDC-mIL-5Rα cells were incubated with baculovirus-expressed mIL-5~biotin overnight. Cytosolic and nuclear fractions were prepared, fractions co-immunoprecipitated with strepavidin-conjugated magnetobeads and samples prepared for SDS-PAGE before immunoblotting with streptavidin~HRP (A). Immunoblots were stripped and reprobed with anti-IL-5Rα antibody (B).

5.6 Discussion

The studies reported here demonstrate the nuclear localization of the full-length IL-5 receptor α subunit using a biochemical approach. Furthermore, the experimental results suggest that this nuclear localization is directly dependent on the presence of IL-5 with changes in nuclear accumulation of the receptor after IL-5 depletion able to be observed within 60 min.

Even though nuclear localization of the receptor had been shown before, no information was available as to whether the nuclear form of the receptor was intact or not since the antibodies used in the previous studies only recognized the intracellular portion of the receptor (Calanni, 1997). The results presented here clearly indicate for the first time that full length receptor can be found in the nucleus, dependent on the presence of IL-5.

In IP experiments, the IL-5R α subunit was co-immunoprecipitated with IL-5. Whilst a clear signal was detected in the cytosolic fraction, the extremely low signal in the nucleus indicates a low amount of IL-5 associated with the receptor, suggesting that after passage into the nucleus, dissociation of the ligand-receptor complex may take

place. This has been shown to be the case before for IL-1, which is not found to be associated with its receptor subsequent to nuclear translocation (Solari *et al.*, 1994).

Interestingly in this context, indirect immunofluorescence studies with the myeloid eosinophil differentiating, AML14.3D10 cell line had indicated that when incubated with the mIL-5 NLS1$^-$ form, the amount of IL-5 receptor α subunit in the nucleus decreased substantially by about 40-60% (Calanni, 1997). Bearing in mind the flow cytometric analysis of the receptor binding capacity of this NLS mutant, which binds the receptor with only low affinity, this is further evidence that nuclear localization of the receptor is directly dependent on its ligand.

Further analysis is needed to clarify the question of the physiological role of nuclear localization of the IL-5 receptor α subunit. It is also important to address the question of whether the observed nuclear transport is indeed IL-5Rα-specific or whether it can be observed for IL-3 and GM-CSF receptor subunits as well.

CHAPTER 6 GENERAL DISCUSSION

IL-5 is an eosinophil differentiation and activating factor. Because eosinophils play a central role in the pathogenesis of asthma and allergic diseases, an understanding of the signal transduction mechanisms specific for IL-5 is of paramount importance. Although the biological activities of IL-5 with respect to eosinophils are well established, the relationship between IL-5 signalling and function is only now starting to emerge. Many intracellular IL-5 receptor-mediated signalling pathways have been elucidated. However, these pathways are not exclusively activated by IL-5, but are shared by IL-3 and GM-CSF, which, together with IL-5 utilize a common signaling entity, the β_c-receptor subunit.

IL-5 appears to possess a functional NLS, indicating that nuclear localization may constitute a novel, ligand-specific pathway through which the distinct effects of IL-5 in eosinophil activation are achieved (Jans *et al.,* 1997ab). This emerging model of a signal transduction mechanism by which specificity is ensured has not received much attention in the field of IL-5 signalling.

Thus, the present study represents the first elucidation of the detailed mechanisms of nuclear localization of IL-5 and its receptor subunits. *In vitro* nuclear transport assays have been utilized to shed light on the mechanisms of IL-5 nuclear transport, whilst the nuclear translocation of both IL-5 and its receptor has been verified in the context of a whole living cell.

The present study revealed that:

1. In addition to the previously identified NLS1 region a newly defined NLS3 region appears to be functional in nuclear transport of hIL-5; IL-5 would appear not to possess a classical bipartite NLS and is not recognized by importin α/β,
2. Nuclear transport of IL-5 itself can occur independently of the conventional nuclear transport machinery,
3. A number of new approaches were developed and used to demonstrate nuclear localization of IL-5,
4. Nuclear localization of intact IL-5 receptor α subunit was shown for the first time to occur in living cells, with nuclear receptor localization dependent on the presence of IL-5.

6.1 The IL-5 Nuclear Localisation Signal

NLSs are defined as sequences necessary and sufficient for nuclear targeting of their respective proteins. Although there is no strict consensus sequence, conventional NLSs typically comprise predominantly basic amino acids (Dingwall and Laskey, 1991) arranged either in a single cluster (monopartite) or in two clusters separated by a spacer of approximately 10-12 amino acids (bipartite). Several studies of "classical type" bipartite NLS have indicated the role of particular residues necessary for nuclear nuclear targeting functionality (Robbins *et al.,* 1991; Hu and Jans, 1999; Efthymiadis *et al.,* 1997).

It has previously been shown that the human IL-5 NLS region is able to target a heterologous protein to the nucleus, with mutations within the proximal arm of the NLS region (NLS1) abolishing the nuclear targeting activity (Jans *et al.,* 1997a). Intriguingly, subsequent experiments with an NLS2 mutant form, in which Arg 108 and Arg 110 of the distal arm were mutated, showed that this region is not essential for cotargeting of the IL-5 receptor subunits to the nucleus mediated by IL-5 (Calanni, 1997).

These results imply that the IL-5 NLS does not represent a conventional bipartite NLS. Analysis of the IL-5 sequence of both mouse and human revealed that a third short cluster of basic amino acid residues (NLS3) is present between the formerly identified NLS1 and NLS2 sequences. The nuclear exclusion of both the IL-5 NLS1 and NLS3 mutant β-Gal fusion proteins as demonstrated in Chapter 3 indicate that NLS3 is integral to hIL-5 NLS function. It is therefore hypothesized that the residues 95-104 in hIL-5 constitute the hIL-5 NLS, implying that the IL-5 NLS may not describe a conventional class of NLS.

Previously, a number of non-conventional NLS have been identified (see Table 6.1). An apparently novel NLS is that of the HIV-1 Tat transactivator protein (G**RKKRR**Q**RRR**AP^{59}; Siomi *et al.,* 1990), which is sufficient to target β-galactosidase (476 kDa) to the nucleus (Efthymiadis *et al.,* 1998). The interferon-induced transcription factor IFI-16 NLS (Q**KRKK**ST**K**E**K**AGPKGSKVSRDW^{145}) confers nuclear transport that requires ATP, but is Ran- and importin α/β-independent (Briggs et al., 2001).

The K-NLS (YD**RR**G**R**PGC**R**YDGMVGFSADETWDSAIDTWSPSEWQMAY^{361}) of the mRNA binding hnRNP K shuttling protein is able to mediate nuclear import in

cytosolic factor-independent, but ATP-dependent fashion (Michael *et al.*, 1997).

Clearly, IL-5 is not unique in possessing a signal-dependent nuclear import pathway that does not utilise importin α/β or Ran. The lack of homology between the IL-5 NLS and the other NLSs listed in Table 6.1, however, implies that the respective NLSs are likely to mediate nuclear import through distinct pathways.

In contrast to the IL-5 wild type form, the NLS1$^-$ mutant of mIL-5 was previously found to show greatly reduced ability to co-transport the soluble IL-5 receptor subunits into the nucleus *in vitro* (Calanni, 1997). Furthermore, *in vitro* eosinophil differentiation assays indicated that the mIL-5 NLS1$^-$ mutant derivative was unable to stimulate the production of eosinophil colonies in bone marrow cultures (unpublished data, Takamoto, Shinshu University School of Medicine, Matsumoto, Japan). The basis of this observation was thought to be that the proximal arm of the IL-5 bipartite NLS was indeed essential for full function through the NLS. The flow cytometric receptor binding experiments performed with the mIL-5 NLS1$^-$ mutant derivative described in Chapter 3 indicate that a more likely reason for this observation is that the reduced binding affinity of the NLS1 mutant to the mIL-5 receptor abolishes receptor cotransport. This of course does not mean that NLS1 does not play a role in nuclear transport; clearly, the experiments with IL-5-NLS-β-Gal support the idea that it does. However, the fact that the NLS1 mutation impairs receptor binding renders general conclusions about its physiological role impossible. For future analysis of receptor binding of IL-5 mutant derivatives prior functional studies, the flow cytometric approach described here utilizing fluorescently labelled ligands represents a simple, reliable and safe alternative to more traditional methods such as binding studies with radiolabelled ligands.

6.2 Pathway of IL-5 NLS conferred nuclear accumulation

Classical NLS-dependent nuclear transport is a multistep process dependent on numerous cytosolic factors including importin α/β and the monomeric GTP-binding protein Ran (see Chapter 1.2.3). The results of the *in vitro* nuclear transport assays with hIL-5 and mIL-5 indicate that their import does not depend on cytosolic factors, ATP or Ran (see Section 3.8). Nuclear import was, however, inhibited by WGA and excess importin β, suggesting that direct interaction with O-glycosylated nucleoporins may

play integral part in the translocation process of IL-5 (see Section 3.9). Similar results were obtained for the hIL-5_{sc}-HSA fusion protein, which was created to exclude the possibility that nuclear import of IL-5, whose size is below the MW cut-off of an NPC, is due to passive diffusion. Nuclear import of the hIL-5_{sc}-HSA fusion protein was independent of cytosolic factors as well as of ATP, and was blocked by WGA. The IL-5 NLS was not recognised by importin α/β in the ELISA assay, indicating that it is not a classical bipartite NLS, consistent with the idea that IL-5 does not utilize a conventional importin α/β-mediated nuclear import pathway.

As mentioned, signal-dependent but importin/Ran independent nuclear import pathways are purported to exist based on results for a diverse group of proteins (Efthymiadis *et al.,* 1998; Fagotto *et al.,* 1999; Michael *et al.,* 1997; Schmalz *et al.,* 1998; Sweitzer and Hanover, 1996), including HIV-1 Tat, β-catenin, hnRNP K, IFI-16 and PKC-α (see Table 6.1).

Clearly, IL-5 is by no means unique in possessing a signal-dependent nuclear import pathway that does not utilise importin α/β or Ran; since the different proteins listed in Table 6.1 are not identical in terms of their nuclear import properties, it seems clear that these are multiple importin-independent nuclear transport pathways awaiting molecular delineation.

So far, little is know about the mechanisms by which other polypeptides and growth factor ligands/receptors are being translocated into the nucleus. Two recent reports for insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 (Schedlich *et al.*, 2000), and fibroblast growth factor receptor (FGFR)1 (Reilly and Maher, 2001), provided first evidence that nuclear transport of polypeptide hormone and growth factor ligands/receptors can be facilitated by importin-mediated mechanisms.

Nuclear translocation in T47D breast cancer cells of IGFBP-3, carrying a putative NLSs within the C-terminal domain, appeared to occur by a nuclear localization sequence (NLS)-dependent pathway mediated by the importin β nuclear transport factor and requiring both ATP and GTP hydrolysis. Both IGFBP-3 and IGFBP-5 were shown to be recognized by importin β and the α/β heterodimer but only poorly by importin α (Schedlich *et al.*, 2000).

Table 6.1. Examples of proteins utilizing novel nuclear import pathways.

Protein	Nuclear import properties*	Reference(s)
β-catenin	direct binding to nuclear envelope independent of importins; requires no ATP/GTP; Ran-independent	Fagotto *et al.* (1998); Yokoya *et al.* (1999)
hnRNP K	NLS:(YD**RR**G**R**PGC**R**YDGMVGFSADETWDSAIDTWSPSEWQMAY^{361}) not recognized by importins; nuclear transport ATP-dependent, but does not require Ran	Michael *et al.* (1997)
IFI-16	NLS: (Q**KRKK**ST**KE**KAGPKGSKVSRDW^{145}); transport requires ATP, but is Ran-independent; importin α/β does not recognize IFI-16 with high affinity; binding to nuclear components mediated by NLS, regulated by CK2 phosphorylation	Briggs *et al.* (2001)
IL-5	hIL-5 NLS: **KK**YIDGQ**KKK**^{105} not recognized by importin α or β; nuclear transport independent of ATP or GTP; WGA and importin β inhibit nuclear accumulation	This study
PKC-α	NLS peptides, nonhydrolysable GTP analogues, WGA and antibodies to importin β do not inhibit nuclear transport; cytoskeleton integrity important	Beckmann *et al.* (1994); Schmalz *et al.* (1998)
HIV-1 Tat	NLS: G**RKKRRQRRR**AP^{59} sufficient to target β-Gal into nucleus in ATP-dependent manner; not recognized by either importin α or β; non-hydrolysable GTP analogues prevent nuclear import;	Efthymiades *et al.* (1998); Siomi *et al.* (1990)
HIV-1 Vpr	does not require Ran-mediated GTP hydrolysis or ATP; transport independent of importin α/β; intranuclear binding depends on cytosolic factors	Jenkins et al. (1998); Subbramanian et al. (1998); Jans et al. (2000);

* Conventional signal-dependent nuclear protein import (see Section 1.2.3) is mediated by importins/Ran/nucleoporins/ATP and therefore inhibited by antibodies to importins, nonhydrolysable GTP analogues as well as WGA, which binds to nucleoporins. Exogenous cytosol is normally required *in vitro* to supply importins and Ran. Binding to nuclear components is not conferred by conventional NLSs.

FGF-2 (18 kD) is known to induce nuclear translocation of the membrane-bound form of FGFR1, despite the lack of an NLS in either the ligand or the receptor (Maher, 1996; see Section 1.3.3). Recently, Reilly and Maher (2001) have shown that nuclear translocation FGFR1 occurs via a mechanism distinct from classical nuclear import but dependent on importin β, apparently in an energy-independent manner.

6.3 Importance of the IL-5 NLS for signalling

Various studies have indicated that polypeptide ligands and growth factors such as FGF (Imamura *et al.,* 1990, Wiedlocha *et al.,* 1994), PDGF (Collins *et al.,* 1987) and IFNγ (Zu and Jay, 1991; Bader and Weitzerbin, 1994) have significant signalling roles mediated by their functional NLS. The identification of a functional NLS within IL-5 thus suggests that its nuclear transport may contribute to signalling. IL-5 may mediate its action by cotransporting signalling molecules to the nucleus where they may interact with chromatin or modulate transcriptional activities by interacting with nuclear transcription factors.

The precise mechanisms by which polypeptide ligands and their receptors traffic from the extracellular space to the interior of a cell after internalization is still not well established. To reach the cytosol and the nucleus from the extracellular space, polypeptide ligands and growth factors must translocate across a cellular membrane, either at the cell surface or across the membrane of an intracellular organelle. The same challenge of crossing a membrane barrier is shared by many protein toxins that bind to cell-surface receptors and exert their effect in the cytosol (Wesche *et al.,* 2000). Diphtheria toxin, for example, binds to the uncleaved precursor of heparin-binding EGF (HB-EGF) at the cell surface and is subsequently endocytosed. Upon exposure to the low pH obtained in endosomes, the A-fragment of the toxin is translocated to the cytosol where it enzymatically modifies elongation factor 2 and thereby inhibits protein synthesis (Naglich *et al.,* 1992ab).

The present study has not addressed the question of how IL-5/IL-5 receptor can escape the lysosomal degradation pathway. Nevertheless, the *in vivo* studies (see Chapter 4) indicate that at least 8% of the IL-5 that is internalized reaches the nucleus within 12 hours. This implies that IL-5, and any associated molecules may localize to the nucleus through a unique pathway that allows exit from the endosomes. This pathway may be inefficient as most of IL-5 is being degraded in lysosomes, but it seems likely that the relatively small amount of ligand in the nucleus is functionally significant.

There is clear evidence that the receptors of many growth factors and cytokines can colocalize in the nucleus after endocytosis (see Table 1.5). This implies that the receptor, alone or in complex with their respective ligands, may have a direct role in nuclear signalling. Previously, piggyback experiments performed with the extracellular

domains of the IL-5 receptor α and β subunits suggested that the IL-5 NLS can specifically cotarget the IL-5 receptor into the nucleus (Jans *et al.,* 1997b; Calanni, 1997). Support for the possibility that receptor co-localisation may be important for IL-5 nuclear signalling was obtained in the cell fractionation studies here which verified that full length, membrane-integral IL-5 receptor α subunit is targeted to the nucleus in intact cells (see Chapter 5). IL-5 and the IL-5 receptor α-subunit not only localized in the cytosolic fraction, but also in the nucleus. These results verify the data from immunofluorescence studies (Jans *et al.,* 1997a; Calanni, 1997), where IL-5 as well as the IL-5 receptor subunits could be detected in both cytoplasm and nucleus. Subcellular localization in the cytoplasm presumably represents both membrane-bound ligand receptor complex as well as complex internalized after endocytosis. Nuclear localization, however, would not be anticipated according to the conventional model of receptor and ligand being degraded in the lysosomal pathway after internalization. That nuclear import of the IL-5 receptor α subunit is drastically reduced in the absence of IL-5, but reaches maximum levels within 1 week after re-addition of IL-5 (see Section 5.4) is clear evidence for ligand dependence of IL-5 receptor nuclear targeting.

Immunoprecipitation experiments using biotinylated IL-5 showed strong association of IL-5 with its α receptor subunit in the cytosolic fraction, but only a small amount of the receptor subunit in the nuclear fraction, suggesting that the ligand/receptor complex may dissociate once it reaches the nuclear compartment of the cell.

In order to verify the importance of the IL-5 NLS regions in piggybacking the IL-5 receptor subunits to the nucleus, it is crucial to perform these fractionation and immunoprecipitation experiments with IL-5 NLS mutant derivatives; the latter, of course, must be completely functional in receptor binding.

The lack of sensitivity of the anti-β_c antibody available meant that it was not possible to assess the nuclear import of this receptor subunit. Since it is shared with IL-3 and GM-CSF, it can be speculated that β_c may not play a role in nuclear signalling of IL-5, though there is no evidence for this as yet. Consistent with this idea, however, Martinez-Moczygemba and Huston (2001) recently showed that agonistic ligation of the IL-3/IL-5/GM-CSF α and β_c receptor subunits rapidly induces proteasomal degradation of the cytoplasmic domain of β_c, but not Rα, although these experiments do not discount the possibility that a small number of receptor molecules might be able to escape degradation. One way to verify nuclear localization of β_c would be to label cell surface

proteins before subfractionation and protein precipitation by biotinylating the cell surfaces of receptor-expressing cells. Taken together the results indicate that IL-5 receptor nuclear translocation can occur, apparently in IL-5-dependent fashion, and that the IL-5 receptor subunits may therefore play a role in nuclear signalling.

6.4 Mechanisms of Nuclear Signalling by IL-5 and its receptor

Specificity can arise from multiple points in a signalling pathway, such as at the level of the receptor, through the modulation of signalling kinetics, through cross-talk between different signalling pathways, or through ligand-specific downstream signalling events. Nuclear localization of polypeptide ligands, as well as their receptors, may represent a more direct, "instructive" signalling mechanism (see Section 1.1.9), guaranteeing the specificity of the response to the particular ligand in the respective target cells.

Within the nucleus, the ligand or ligand/receptor complex may induce changes by binding to DNA or chromatin, activating nuclear kinases or acting as/associating with transcription factors. Chromatin binding properties have been observed for a number of growth factors as well as their receptors, such as Angiotensin II (Re *et al.,* 1984ab), NGF (Rakowicz-Szulczynska *et al.,* 1986b), EGF (Lin *et al.,* 2001; Rakowicz-Szulczynska *et al.,* 1986b), PDGF (Rakowicz-Szulczynska *et al.,* 1986b), SDGF (Kimura, 1993) and GH (Lobie *et al.,* 1991) (see Table 1.5).

It was previously shown that the removal of a putative NLS in the N terminus of cyclophilin B (CypB), a peptidylprolyl isomerase (PPI) found in the endoplasmic reticulum, extracellular space as well as in the nucleus, abrogated the potentiation in prolactin (PRL)-induced proliferation of PRL-dependent cell lines and the enhancement of PRL nuclear transport, implying a necessary role for CypB in these functions (Rycyzyn *et al.*, 2000). Most recently, a report by Rycyzyn and Clevenger (2002) indicated that the intranuclear PRL/CypB complex could act as a transcriptional inducer by directly interacting with the trancriptional activator Stat5. The enhancement of Stat5-mediated gene trancription by the PRL/CypB protein complex was associated with increased Stat5 DNA-binding activity, resulting from the release of the repressor protein PIAS3 (protein inhibitor of activated Stat3), demonstrating how an intranuclear polypeptide hormone can potentiate its own signal and possibly contribute to its own signalling specificitiy (Rycyzyn and Clevenger, 2002).

The binding of angiotensin II to chromatin was found to enhance the susceptibility of chromatin to nuclease digestion, implying induction of transcriptional activity (Re *et al.*, 1984ab), whilst chromatin binding of NGF resulted in a decrease in transcriptional activity (Rakowicz-Szulczynska *et al.*, 1986b). The EGF receptor also binds chromatin after nuclear localization in target cells (Rakowicz-Szulczynska *et al.*, 1989), and it has recently been demonstrated to bind and activate AT-rich consensus-sequence-dependent transcription, including the consensus site of the cyclin D1 promoter region, thus acting as a potential transcription factor (Lin *et al.*, 2001). SDGF, which requires nuclear localization to induce a mitogenic response in target cells, has also been shown to bind to AT-rich DNA sequences (Kimura *et al.*, 1993). Rao *et al.* (1995b) have shown that the PRL receptor associates with purified nuclear chromatin, whilst chromatin accumulation has also been observed for IFN-α and –β *in vitro* (Kushnaryov *et al.*, 1985; MacDonald *et al.*, 1986).

In showing piggyback localisation of the IL-5 receptors through IL-5, Calanni (1997) concluded that the IL-5 receptor complex is unlikely to directly interact with chromatin or DNA, as nuclear accumulation does not appear to occur through binding to nuclear components. In contrast, there appeared to be some evidence that IL-5 itself, independent of receptor subunits, may accumulate in the nucleus through binding to nuclear components (Calanni, 1997), although the present study does not support this idea (see Section 3.9).

Tavernier *et al.* (2000) recently showed that IL-5, but not IL-3 or GM-CSF can regulate the isoform expression of the IL-5 receptor α subunit. Interestingly, in the context of the role of IL-5 in the nucleus, the kinetics of switching from soluble to transmembrane IL-5Rα was very slow, with a maximal effect after approximately 2 weeks. The molecular mechanisms of this specific action have not been investigated, but it was argued that direct DNA binding of IL-5 could explain this IL-5-specific action. An alternative explanation could be that, while IL-5 is co-translocating its receptor subunits into the nucleus, this could allow the α receptor itself to interact, either directly or indirectly, with its own promoter regions. These sites have been defined (Sun *et al.*, 1996; Zhang *et al.*, 1997), but the factors controlling the transcription of IL-5Rα remain to be established. The present study indicates that the time frame for maximal nuclear accumulation of IL-5Rα after IL-5 stimulation (see Fig. 5.3) is comparable to that of the switch between the two receptor types, thus consistent with the idea that the α receptor

may play a more direct role within the nucleus in this process of reversible switching between membrane-anchored and soluble isoform of IL-5Rα.

Phosphorylation has been reported for FGF-1 receptor after binding of FGF-2 in the nucleus, an event involved in the regulation of the nuclear matrix environment, possibly leading to modulation of transcription, replication and RNA processing (Stachowiak *et al.,* 1996b). Nuclear FGF-2 has been shown to interact directly with Casein kinase 2 (CK2), a serine-threonine kinase (Bonnet *et al.,* 1996), and to stimulate CK2 activity toward nucleolin, a major non-histone nucleolar protein (Bouche *et al.,* 1987; Bonnet *et al.,* 1996).

In a similar manner, insulin seems to stimulate phosphorylation of nucleolin, resulting in RNA efflux from isolated nuclei (Csermely *et al.,* 1993); dephosphorylation of a 30-kDa DNA-binding protein upon incubation of nuclei with activated insulin receptor has also been reported (Gletsu *et al.,* 1999). Possible targets for cytokine receptor action include proteins such as STATs or Grb2 (Mertanie *et al.,* 1999).

An interesting new hypothesis to explain nuclear transport of NLS-containing cytokines as an important part of intracellular signalling networks is that they may act as chaperones to facilitate the nuclear transport of non-NLS-containing proteins associated with the cytokine ligand/receptor complex such as STATs and JAKs (see Section 1.1.7). Subramaniam *et al.* (2000) showed that the NLS of IFNγ, which is required for full biological activity, plays an integral intracellular role in the nuclear translocation of the transcription factor STAT1α activated by IFNγ. Treatment of IFNγ with antibodies to the C-terminal region (95-133) containing the NLS prevented the induction of STAT1α nuclear translocation. A deletion mutant of human IFNγ, lacking the C-terminal NLS region was found to be biologically inactive and, though still able to bind to the IFNγ receptor complex on cells, the NLS mutation specifically abolished the ability IFNγ to initiate STAT1α nuclear translocation.

Thus far, nothing is known about IL-5/IL-5R-associated proteins into the nucleus, but it is possible that JAK2 or Lyn, tyrosine kinases associated with the IL-5 receptor α subunit (Stafford *et al.,* 2002), can be targeted to the nucleus in association with the IL-5 receptor complex.

Novel techniques such as chromatin immunoprecipitation (CHIP) assay as well as cyclic amplification and selection of targets (CASTing) as used for the elucidation of the nuclear role of the EGF receptor (Lin *et al.,* 2001) will be most helpful in identifying

putative DNA sequences recognized by specifically IL-5/IL-5R complexes in association with additional unknown factors subsequent to nuclear localization *in vivo*. The determination of potential interactions between IL-5, IL-5R and DNA binding proteins will undoubtedly assist in the elucidating the role of IL-5 in IL-5-specific gene activation. Electrophoretic mobility shift assays (EMSA), using biotinylated IL-5 and nuclear extracts of IL-5 receptor expressing cell lines may yield the first experimental evidence for the existence of such interactions. Another powerful approach for screening potential binding partners could be to perform yeast two-hybrid screening, utilizing a full-length, single-chain IL-5 open reading frame (ORF) as a Gal4-DNA binding domain (DBD) fusion protein as a bait. Additionally, immunoprecipitation studies in conjunction with subcellular fractionation, involving antibodies directed against proteins known to be associated with the IL-5 receptor system, with subsequent 2-dimensional gel electrophoresis and mass spectroscopical identification of potentially interacting proteins should shed light on this intriguing question.

6.5 Evolution of polypeptide ligand nuclear transport

Pederson (1998) argues that there is rarely anything to lose from thinking in the context of evolution. Growth factors most likely arose with the advent of metazoan life, possibly evolving from ancient chemoattracting ligands in step with co-emerging cell surface receptors. Conceivably, some of today's secreted polypeptide ligands and growth factors may have originated in the course of evolution from proteins that were once capable of triggering cellular responses like growth, adhesion or movement from within (Pederson, 1998). Possibly one of the best examples of such a transition is the FGF ligand family, with its high molecular weight forms still acting in an intracrine manner, while the low molecular weight form is secreted and acts predominantly on the cell surface of FGF-receptor bearing cells.

In the single cell predecessors of metazoans, it is likely that intracellular signal proteins had the function of targeting growth-promoting genes within the genome. Whilst the present day growth factors have largely converted to be signalling molecules primarily functioning on the cell surface by binding to their respective cell surface receptor systems, some of them may simultaneously retain the ability to mediate ligand-specific pathways by direct action in the nucleus (Pederson, 1998).

The presence of polypeptide growth factors and cytokines in the nucleus/nucleolus currently represents an intellectual way station, neither an established piece of orthodoxy on the one hand nor necessarily an opaque box on the other (Pederson, 1998), but with the elucidation of the direct role of the EGFR as a transcription factor involved in regulation of proliferation the field of nuclear import of polypeptide growth factors has gained new experimental support. Importantly, the work with EGFR provides a plausible mechanism for nuclear action by EGF.

Comparable evidence is being accumulated at the moment for a range of polypeptide hormones and their receptors. It remains to be seen whether IL-5 will prove to be another example of a polypeptide ligand exhibiting nuclear transport to achieve full signalling activity, although this seems to be likely, based on the work presented here and elsewhere (Jans *et al.,* 1997ab; Calanni, 1997; Tavernier, 2000). If it does, this should further contribute to the growing idea that polypeptide ligand/receptor nuclear import is a key means of activating **specific** growth factor and cytokine signalling in the physiological context of the multiple receptors, kinase signalling cascades, and transcription factors shared by a myriad of different cytokines and growth factors within a cell.

BIBLIOGRAPHY

Abe, H., R. Nagaoka *et al.* (1993). "Cytoplasmic localization and nuclear transport of cofilin in cultured myotubes." *Exp Cell Res* **206**(1): 1-10.

Adachi, T. and R. Alam (1998). "The mechanism of IL-5 signal transduction." *American Journal of Physiology* **275**(3 Pt 1): C623-33.

Adam, E. J. and S. A. Adam (1994). "Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope." *J Cell Biol* **125**(3): 547-55.

Adam, S. A. and L. Gerace (1991). "Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import." *Cell* **66**(5): 837-47.

Adam, S. A., R. S. Marr *et al.* (1990). "Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors." *J Cell Biol* **111**(3): 807-16.

Adam, S. A., R. Sterne-Marr *et al.* (1992). "Nuclear protein import using digitonin-permeabilized cells." *Methods Enzymol* **219**: 97-110.

Agutter, P. S. and D. Prochnow (1994). "Nucleocytoplasmic transport." *Biochem J* **300**(Pt 3): 609-18.

Altin, J. G. and E. B. Pagler (1995). "A one-step procedure for biotinylation and chemical cross-linking of lymphocyte surface and intracellular membrane-associated molecules." *Anal Biochem* **224**(1): 382-9.

Amalric, F., G. Bouche *et al.* (1994). "Fibroblast growth factor-2 (FGF-2) in the nucleus: translocation process and targets." *Biochemical Pharmacology* **47**(1): 111-5.

Andres, R. Y., I. Jeng *et al.* (1977). "Nerve growth factor receptors: identification of distinct classes in plasma membranes and nuclei of embryonic dorsal root neurons." *Proc Natl Acad Sci U S A* **74**(7): 2785-9.

Antoine, M., K. Reimers *et al.* (1997). "Fibroblast growth factor 3, a protein with dual subcellular localization, is targeted to the nucleus and nucleolus by the concerted action of two nuclear localization signals and a nucleolar retention signal." *J Biol Chem* **272**(47): 29475-81.

Arai, K. I., F. Lee *et al.* (1990). "Cytokines: coordinators of immune and inflammatory responses." *Annu Rev Biochem* **59**: 783-836.

Azuma, C., T. Tanabe *et al.* (1986). "Cloning of cDNA for human T-cell replacing factor (interleukin-5) and comparison with the murine homologue." *Nucleic Acids Res* **14**(22): 9149-58.

Bader, T. and J. Weitzerbin (1994). "Nuclear accumulation of interferon gamma." *Proc Natl Acad Sci U S A* **91**(25): 11831-5.

Bajetto, A., G. Schettini *et al.* (1999). "Nuclear localization of ciliary neurotrophic factor in glial cells." *Brain Res* **818**(2): 565-9.

Bajetto, A., S. Barbero *et al.* (2000). "Immunofluorescence and biochemical techniques to detect nuclear localization of ciliary neurotrophic factor in glial cells." *Brain Res Brain Res Protoc* **5**(3): 273-81.

Balasundaram, D., M. J. Benedik *et al.* (1999). "Nup124p is a nuclear pore factor of Schizosaccharomyces pombe that is important for nuclear import and activity of retrotransposon Tf1." *Mol Cell Biol* **19**(8): 5768-84.

Baldin, V., A. M. Roman *et al.* (1990). "Translocation of bFGF to the nucleus is G1 phase cell cycle specific in bovine aortic endothelial cells." *Embo J* **9**(5): 1511-7.

Baumruker, T., R. Csonga *et al.* (1999). "TNF-alpha and IL-5 gene induction in IgE plus antigen-stimulated mast cells require common and distinct signaling pathways." *Int Arch Allergy Immunol* **118**(2-4): 108-11.

Bayliss, R., T. Littlewood *et al.* (2000). "Structural basis for the interaction between FxFG nucleoporin repeats and importin-beta in nuclear trafficking." *Cell* **102**(1): 99-108.

Bazan, J. F. (1990a). "Structural design and molecular evolution of a cytokine receptor superfamily." *Proc Natl Acad Sci U S A* **87**(18): 6934-8.

Bazan, J. F. (1990b). "Haemopoietic receptors and helical cytokines." *Immunol Today* **11**(10): 350-4.

Beals, C. R., N. A. Clipstone *et al.* (1997). "Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin- sensitive intramolecular interaction." *Genes Dev* **11**(7): 824-34.

Beckmann, R., C. Lindschau *et al.* (1994). "Differential nuclear localization of protein kinase C isoforms in neuroblastoma x glioma hybrid cells." *Eur J Biochem* **222**(2): 335-43.

Bhattacharya, S., B. A. Stout *et al.* (2001). "Granulocyte macrophage colony-stimulating factor and interleukin-5 activate STAT5 and induce CIS1 mRNA in human peripheral blood eosinophils." *Am J Respir Cell Mol Biol* **24**(3): 312-6.

Bikfalvi, A., S. Klein *et al.* (1997). "Biological roles of fibroblast growth factor-2." *Endocr Rev* **18**(1): 26-45.

Blank, V., P. Kourilsky *et al.* (1991). "Cytoplasmic retention, DNA binding and processing of the NF-kappa B p50 precursor are controlled by a small region in its C-terminus." *Embo J* **10**(13): 4159-67.

Blobel, G. and R. W. Wozniak (2000). "Proteomics for the pore." *Nature* **403**(6772): 835-6.

Blumenthal, S. G., G. Aichele *et al.* (1999). "Regulation of the human interleukin-5 promoter by Ets transcription factors. Ets1 and Ets2, but not Elf-1, cooperate with GATA3 and HTLV-I Tax1." *J Biol Chem* **274**(18): 12910-6.

Boilly, B., A. S. Vercoutter-Edouart *et al.* (2000). "FGF signals for cell proliferation and migration through different pathways." *Cytokine Growth Factor Rev* **11**(4): 295-302.

Bonner, W. M. (1975). "Protein migration into nuclei. II. Frog oocyte nuclei accumulate a class of microinjected oocyte nuclear proteins and exclude a class of microinjected oocyte cytoplasmic proteins." *J Cell Biol* **64**(2): 431-7.

Bonnet, H., O. Filhol *et al.* (1996). "Fibroblast growth factor-2 binds to the regulatory beta subunit of CK2 and directly stimulates CK2 activity toward nucleolin." *J Biol Chem* **271**(40): 24781-7.

Boonstra, J., P. Rijken *et al.* (1995). "The epidermal growth factor." *Cell Biol Int* **19**(5): 413-30.

Bottger, A. and B. A. Spruce (1995). "Proenkephalin is a nuclear protein responsive to growth arrest and differentiation signals." *J Cell Biol* **130**(6): 1251-62.

Bouche, G., N. Gas *et al.* (1987). "Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing G0----G1 transition." *Proc Natl Acad Sci U S A* **84**(19): 6770-4.

Bouche, G., V. Baldin *et al.* (1994). "Activation of rDNA transcription by FGF-2: key role of protein kinase CKII." *Cell Mol Biol Res* **40**(5-6): 547-54.

Bozza, P. T., H. C. Castro-Faria-Neto *et al.* (1994). "IL-5 accounts for the mouse pleural eosinophil accumulation triggered by antigen but not by LPS." *Immunopharmacology* **27**(2): 131-6.

Bradding, P., I. H. Feather *et al.* (1993). "Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation." *J Immunol* **151**(7): 3853-65.

Breeuwer, M. and D. S. Goldfarb (1990). "Facilitated nuclear transport of histone H1 and other small nucleophilic proteins." *Cell* **60**(6): 999-1008.

Briggs, L. J., R. W. Johnstone *et al.* (2001). "Novel properties of the protein kinase CK2-site-regulated nuclear- localization sequence of the interferon-induced nuclear factor IFI 16." *Biochem J* **353**(Pt 1): 69-77.

Bruijnzeel, P. L., P. H. Kuijper *et al.* (1993). "The involvement of eosinophils in the patch test reaction to aeroallergens in atopic dermatitis: its relevance for the pathogenesis of atopic dermatitis." *Clin Exp Allergy* **23**(2): 97-109.

Buijs, J., M. W. Egbers *et al.* (1995). "Toxocara-induced eosinophilic inflammation. Airway function and effect of anti-IL-5." *Am J Respir Crit Care Med* **151**(3 Pt 1): 873-8.

Burwen, S. J., M. E. Barker *et al.* (1984). "Transport of epidermal growth factor by rat liver: evidence for a nonlysosomal pathway." *Journal of Cell Biology* **99**(4 Pt 1): 1259-65.

Burwen, S. and A. Jones (1987). "The association of polypeptide hormones and growth factors with the nuclei of target cells." *Trends in Biochemical Sciences* **12**: 159-162.

Buss, F., H. Kent *et al.* (1994). "Role of different domains in the self-association of rat nucleoporin p62." *J Cell Sci* **107**(Pt 2): 631-8.

Busse, W. W. and R. F. Lemanske, Jr. (2001). "Asthma." *N Engl J Med* **344**(5): 350-62.

Butterworth, A. E. (1984). "Cell-mediated damage to helminths." *Adv Parasitol* **23**: 143-235.

Calanni, S. T. (1997). *Nuclear Signalling by Interleukin-5*. JCSMR. Australian National University. Canberra

Campbell, H. D., C. J. Sanderson *et al.* (1988). "Isolation, structure and expression of cDNA and genomic clones for murine eosinophil differentiation factor. Comparison with other eosinophilopoietic lymphokines and identity with interleukin-5." *Eur J Biochem* **174**(2): 345-52.

Cao, Y., M. Ekstrom *et al.* (1993). "Characterization of the nuclear translocation of acidic fibroblast growth factor." *Journal of Cell Science* **104 (Pt 1)**: 77-87.

Carr, P. D., S. E. Gustin *et al.* (2001). "Structure of the complete extracellular domain of the common beta subunit of the human GM-CSF, IL-3, and IL-5 receptors reveals a novel dimer configuration." *Cell* **104**(2): 291-300.

Castro, J. M. and M. G. Barcia (1996). "Localization of prothymosin alpha in the nucleus." *Biochem Biophys Res Commun* **224**(1): 140-6.

Catimel, B., T. Teh *et al.* (2001). "Biophysical characterization of interactions involving importin-alpha during nuclear import." *J Biol Chem* **276**(36): 34189-98.

Chaiken, I. M. and W. V. Williams (1996). "Identifying structure-function relationships in four-helix bundle cytokines: towards de novo mimetics design." *Trends Biotechnol* **14**(10): 369-75.

Chand, N., J. E. Harrison *et al.* (1992). "Anti-IL-5 monoclonal antibody inhibits allergic late phase bronchial eosinophilia in guinea pigs: a therapeutic approach." *Eur J Pharmacol* **211**(1): 121-3.

Chelsky, D., R. Ralph *et al.* (1989). "Sequence requirements for synthetic peptide-mediated translocation to the nucleus." *Mol Cell Biol* **9**(6): 2487-92.

Chen, R., Y. V. Mukhin *et al.* (2000). "A functional angiotensin II receptor-GFP fusion protein: evidence for agonist-dependent nuclear translocation [In Process Citation]." *Am J Physiol Renal Physiol* **279**(3): F440-8.

Chi, N. C. and S. A. Adam (1997). "Functional domains in nuclear import factor p97 for binding the nuclear localization sequence receptor and the nuclear pore." *Mol Biol Cell* **8**(6): 945-56.

Chida, K. and P. K. Vogt (1992). "Nuclear translocation of viral Jun but not of cellular Jun is cell cycle dependent." *Proc Natl Acad Sci U S A* **89**(10): 4290-4.

Chihara, J., J. Plumas *et al.* (1990). "Characterization of a receptor for interleukin 5 on human eosinophils: variable expression and induction by granulocyte/macrophage colony-stimulating factor." *J Exp Med* **172**(5): 1347-51.

Clevenger, C. V., D. H. Russell *et al.* (1990a). "Regulation of interleukin 2-driven T-lymphocyte proliferation by prolactin." *Proc Natl Acad Sci U S A* **87**(16): 6460-4.

Clevenger, C. V., A. L. Sillman *et al.* (1990b). "Interleukin-2 driven nuclear translocation of prolactin in cloned T-lymphocytes." *Endocrinology* **127**(6): 3151-9.

Clevenger, C. V., S. W. Altmann *et al.* (1991). "Requirement of nuclear prolactin for interleukin-2-stimulated proliferation of T lymphocytes." *Science* **253**(5015): 77-9.

Clevenger, C. V. and M. A. Rycyzyn (2000). "Translocation and action of polypeptide hormones within the nucleus. Relevance to lactogenic transduction." *Adv Exp Med Biol* **480**: 77-84.

Clever, J. and H. Kasamatsu (1991). "Simian virus 40 Vp2/3 small structural proteins harbor their own nuclear transport signal." *Virology* **181**(1): 78-90.

Clinton, M., L. Graeve *et al.* (1991). "Evidence for nuclear targeting of prothymosin and parathymosin synthesized in situ." *Proc Natl Acad Sci U S A* **88**(15): 6608-12.

Clutterbuck, E., J. G. Shields *et al.* (1987). "Recombinant human interleukin 5 is an eosinophil differentiation factor but has no activity in standard human B cell growth factor assays." *Eur J Immunol* **17**(12): 1743-50.

Coffer, P. J., R. C. Schweizer *et al.* (1998). "Analysis of signal transduction pathways in human eosinophils activated by chemoattractants and the T-helper 2-derived cytokines interleukin-4 and interleukin-5." *Blood* **91**(7): 2547-57.

Collins, T., D. T. Bonthron *et al.* (1987). "Alternative RNA splicing affects function of encoded platelet-derived growth factor A chain." *Nature* **328**(6131): 621-4.

Conti, E., M. Uy *et al.* (1998). "Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha." *Cell* **94**(2): 193-204.

Conti, E. and J. Kuriyan (2000). "Crystallographic analysis of the specific yet versatile recognition of distinct nuclear localization signals by karyopherin alpha." *Structure Fold Des* **8**(3): 329-38.

Cordes, V. C., S. Reidenbach *et al.* (1995). "High content of a nuclear pore complex protein in cytoplasmic annulate lamellae of Xenopus oocytes." *Eur J Cell Biol* **68**(3): 240-55.

Csaba, G., F. Sudar *et al.* (1983). "Comparative study of the internalization and nuclear localization of amino acid type hormones in Tetrahymena and rat lymphocytes." *Exp Clin Endocrinol* **82**(1): 61-7.

Csermely, P., T. Schnaider *et al.* (1993). "Insulin induces the phosphorylation of nucleolin. A possible mechanism of insulin-induced RNA efflux from nuclei." *J Biol Chem* **268**(13): 9747-52.

Csonga, R., E. E. Prieschl *et al.* (1998). "Common and distinct signaling pathways mediate the induction of TNF-alpha and IL-5 in IgE plus antigen-stimulated mast cells." *J Immunol* **160**(1): 273-83.

Curtis, B. M., M. B. Widmer *et al.* (1990). "IL-1 and its receptor are translocated to the nucleus." *Journal of Immunology* **144**(4): 1295-303.

Dabauvalle, M. C., B. Schulz *et al.* (1988). "Inhibition of nuclear accumulation of karyophilic proteins in living cells by microinjection of the lectin wheat germ agglutinin." *Exp Cell Res* **174**(1): 291-6.

Damelin, M. and P. A. Silver (2000). "Mapping interactions between nuclear transport factors in living cells reveals pathways through the nuclear pore complex." *Mol Cell* **5**(1): 133-40.

Darnell, J. E., Jr. (1997). "STATs and gene regulation." *Science* **277**(5332): 1630-5.

Daser, A., K. Koetz *et al.* (2000). "Genetics of atopy in a mouse model: polymorphism of the IL-5 receptor alpha chain." *Immunogenetics* **51**(8-9): 632-8.

Davis, L. I. (1995). "The nuclear pore complex." *Annu Rev Biochem* **64**: 865-96.

de Groot, R. P., T. B. van Dijk *et al.* (1997). "Activation of 12-O-tetradecanoylphorbol-13-acetate response element- and dyad symmetry element-dependent transcription by interleukin-5 is mediated by Jun N-terminal kinase/stress-activated protein kinase kinases." *J Biol Chem* **272**(4): 2319-25.

de Groot, R. P., P. J. Coffer *et al.* (1998). "Regulation of proliferation, differentiation and survival by the IL-3/IL-5/GM-CSF receptor family." *Cellular Signalling* **10**(9): 619-28.

de Miguel, F., N. Fiaschi-Taesch *et al.* (2001). "The C-terminal region of PTHrP, in addition to the nuclear localization signal, is essential for the intracrine stimulation of proliferation in vascular smooth muscle cells." *Endocrinology* **142**(9): 4096-105.

Defize, L. H., W. H. Moolenaar *et al.* (1986). "Dissociation of cellular responses to epidermal growth factor using anti-receptor monoclonal antibodies." *Embo J* **5**(6): 1187-92.

del Peso, L., M. Gonzalez-Garcia *et al.* (1997). "Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt." *Science* **278**(5338): 687-9.

Delrieu, I., E. Arnaud *et al.* (1998). "Overexpression of the FGF-2 24-kDa isoform up-regulates IL-6 transcription in NIH-3T3 cells." *FEBS Lett* **436**(1): 17-22.

Delrieu, I. (2000). "The high molecular weight isoforms of basic fibroblast growth factor (FGF-2): an insight into an intracrine mechanism." *FEBS Lett* **468**(1): 6-10.

Dent, L. A., M. Strath *et al.* (1990). "Eosinophilia in transgenic mice expressing interleukin 5." *J Exp Med* **172**(5): 1425-31.

Devos, R., G. Plaetinck *et al.* (1995). "Interleukin-5 and its receptor: a drug target for eosinophilia associated with chronic allergic disease." *Journal of Leukocyte Biology* **57**(6): 813-9.

Dewson, G., G. M. Walsh *et al.* (1999). "Expression of Bcl-2 and its homologues in human eosinophils. Modulation by interleukin-5." *Am J Respir Cell Mol Biol* **20**(4): 720-8.

Dibbert, B., I. Daigle *et al.* (1998). "Role for Bcl-xL in delayed eosinophil apoptosis mediated by granulocyte-macrophage colony-stimulating factor and interleukin-5." *Blood* **92**(3): 778-83.

Dickason, R. R. and D. P. Huston (1996). "Creation of a biologically active interleukin-5 monomer." *Nature* **379**(6566): 652-5.

Dingwall, C., S. M. Dilworth *et al.* (1987). "Nucleoplasmin cDNA sequence reveals polyglutamic acid tracts and a cluster of sequences homologous to putative nuclear localization signals." *Embo J* **6**(1): 69-74.

Dingwall, C. and R. A. Laskey (1991). "Nuclear targeting sequences--a consensus?" *Trends Biochem Sci* **16**(12): 478-81.

Djabali, K., V. M. Aita *et al.* (2001). "Hairless is translocated to the nucleus via a novel bipartite nuclear localization signal and is associated with the nuclear matrix." *J Cell Sci* **114**(Pt 2): 367-76.

Dubucquoi, S., P. Desreumaux *et al.* (1994). "Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion." *J Exp Med* **179**(2): 703-8.

Dvorak, A. M., H. Saito *et al.* (1989). "Ultrastructure of eosinophils and basophils stimulated to develop in human cord blood mononuclear cell cultures containing recombinant human interleukin-5 or interleukin-3." *Lab Invest* **61**(1): 116-32.

Dworetzky, S. I., R. E. Lanford *et al.* (1988). "The effects of variations in the number and sequence of targeting signals on nuclear uptake." *J Cell Biol* **107**(4): 1279-87.

Efthymiadis, A., H. Shao *et al.* (1997). "Kinetic characterization of the human retinoblastoma protein bipartite nuclear localization sequence (NLS) in vivo and in vitro. A comparison with the SV40 large T-antigen NLS." *J Biol Chem* **272**(35): 22134-9.

Efthymiadis, A., L. J. Briggs *et al.* (1998). "The HIV-1 Tat nuclear localization sequence confers novel nuclear import properties." *J Biol Chem* **273**(3): 1623-8.

Eguchi, H., T. Ikuta *et al.* (1997). "A nuclear localization signal of human aryl hydrocarbon receptor nuclear translocator/hypoxia-inducible factor 1beta is a novel bipartite type recognized by the two components of nuclear pore- targeting complex." *J Biol Chem* **272**(28): 17640-7.

Ema, H., T. Suda *et al.* (1990). "Target cells for granulocyte colony-stimulating factor, interleukin-3, and interleukin-5 in differentiation pathways of neutrophils and eosinophils." *Blood* **76**(10): 1956-61.

Enkemann, S. A., R. D. Ward *et al.* (2000). "Mobility within the nucleus and neighboring cytosol is a key feature of prothymosin-alpha." *J Histochem Cytochem* **48**(10): 1341-55.

Everett, A. D., T. Stoops *et al.* (2001). "Nuclear targeting is required for hepatoma-derived growth factor-stimulated mitogenesis in vascular smooth muscle cells." *J Biol Chem* **276**(40): 37564-8.

Fabre, E. and E. Hurt (1997). "Yeast genetics to dissect the nuclear pore complex and nucleocytoplasmic trafficking." *Annu Rev Genet* **31**: 277-313.

Fabre, E. and E. C. Hurt (1994). "Nuclear transport." *Curr Opin Cell Biol* **6**(3): 335-42.

Fagotto, F., U. Gluck *et al.* (1998). "Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin." *Curr Biol* **8**(4): 181-90.

Fanara, P., M. R. Hodel *et al.* (2000). "Quantitative Analysis of Nuclear Localization Signal (NLS)-Importin alpha Interaction through Fluorescence Depolarization. Evidence for auto-inhibitory regulation of NLS binding." *J Biol Chem* **275**(28): 21218-21223.

Featherstone, C., M. K. Darby *et al.* (1988). "A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA in vivo." *J Cell Biol* **107**(4): 1289-97.

Feldherr, C. M. (1962). "The nuclear annuli as pathways for nucleocytoplasmic exchanges." *J Cell Biol* **14**: 65-72.

Feldherr, C. M., E. Kallenbach *et al.* (1984). "Movement of a karyophilic protein through the nuclear pores of oocytes." *J Cell Biol* **99**(6): 2216-22.

Filla, M. S., P. Dam *et al.* (1998). "The cell surface proteoglycan syndecan-1 mediates fibroblast growth factor-2 binding and activity." *J Cell Physiol* **174**(3): 310-21.

Finlay, D. R. and D. J. Forbes (1990). "Reconstitution of biochemically altered nuclear pores: transport can be eliminated and restored." *Cell* **60**(1): 17-29.

Florkiewicz, R. Z. and A. Sommer (1989). "Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons." *Proc Natl Acad Sci U S A* **86**(11): 3978-81.

Florkiewicz, R. Z., J. Anchin *et al.* (1998). "The inhibition of fibroblast growth factor-2 export by cardenolides implies a novel function for the catalytic subunit of Na+,K+-ATPase." *J Biol Chem* **273**(1): 544-51.

Folkard, S. G., M. J. Taylor *et al.* (1997). "Protective responses against skin-dwelling microfilariae of Onchocerca lienalis in severe combined immunodeficient mice." *Infect Immun* **65**(7): 2846-51.

Fontes, M. R., T. Teh *et al.* (2000). "Structural basis of recognition of monopartite and bipartite nuclear localization sequences by mammalian importin-alpha." *J Mol Biol* **297**(5): 1183-94.

Forwood, J. K. (2001). *Importin-mediated nuclear import pathways for DNA-binding proteins*. JCSMR. Australian National University. Canberra

Franke, T. F., D. R. Kaplan *et al.* (1997). "PI3K: downstream AKTion blocks apoptosis." *Cell* **88**(4): 435-7.

Freire, J., G. Covelo *et al.* (2001). "Identification of nuclear-import and cell-cycle regulatory proteins that bind to prothymosin alpha." *Biochem Cell Biol* **79**(2): 123-31.

Fridell, R. A., R. Truant *et al.* (1997). "Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-beta." *J Cell Sci* **110**(Pt 11): 1325-31.

Friedman, S., X. Zhan *et al.* (1994). "Mutagenesis of the nuclear localization sequence in EGF-1 alters protein stability but not mitogenic activity." *Biochem Biophys Res Commun* **198**(3): 1203-8.

Garre, C., G. Bianchi-Scarra *et al.* (1992). "Lactoferrin binding sites and nuclear localization in K562(S) cells." *J Cell Physiol* **153**(3): 477-82.

Geijsen, N., M. Spaargaren *et al.* (1999). "Association of RACK1 and PKCbeta with the common beta-chain of the IL-5/IL-3/GM-CSF receptor." *Oncogene* **18**(36): 5126-30.

Geijsen, N., I. J. Uings *et al.* (2001). "Cytokine-specific transcriptional regulation through an IL-5Ralpha interacting protein." *Science* **293**(5532): 1136-8.

Gletsu, N., W. Dixon *et al.* (1999). "Insulin receptor at the mouse hepatocyte nucleus after a glucose meal induces dephosphorylation of a 30-kDa transcription factor and a concomitant increase in malic enzyme gene expression." *J Nutr* **129**(12): 2154-61.

Goffin, V. and P. A. Kelly (1996). "Prolactin and growth hormone receptors." *Clin Endocrinol (Oxf)* **45**(3): 247-55.

Goldfine, I. D., G. J. Smith *et al.* (1977). "Cellular uptake and nuclear binding of insulin in human cultured lymphocytes: evidence for potential intracellular sites of insulin action." *Proc Natl Acad Sci U S A* **74**(4): 1368-72.

Goldfine, I. D. (1981). "Interaction of insulin, polypeptide hormones, and growth factors with intracellular membranes." *Biochim Biophys Acta* **650**(1): 53-67.

Goldfine, I. D., F. Purrello *et al.* (1982). "Insulin binding sites on the nuclear envelope: potential relationship to mRNA metabolism." *J Cell Biochem* **20**(1): 29-39.

Görlich, D., F. Vogel *et al.* (1995). "Distinct functions for the two importin subunits in nuclear protein import." *Nature* **377**(6546): 246-8.

Görlich, D., P. Henklein *et al.* (1996a). "A 41 amino acid motif in importin-alpha confers binding to importin-beta and hence transit into the nucleus." *Embo J* **15**(8): 1810-7.

Görlich, D., R. Kraft *et al.* (1996b). "Importin provides a link between nuclear protein import and U snRNA export." *Cell* **87**(1): 21-32.

Görlich, D. and U. Kutay (1999). "Transport between the cell nucleus and the cytoplasm." *Annu Rev Cell Dev Biol* **15**: 607-60.

Görlich, D. and I. W. Mattaj (1996c). "Nucleocytoplasmic transport." *Science* **271**(5255): 1513-8.

Görlich, D., N. Pante *et al.* (1996d). "Identification of different roles for RanGDP and RanGTP in nuclear protein import." *Embo J* **15**(20): 5584-94.

Govind, S., E. Drier *et al.* (1996). "Regulated nuclear import of the Drosophila rel protein dorsal: structure-function analysis." *Mol Cell Biol* **16**(3): 1103-14.

Green, I., C. H. Kirkpatrick *et al.* (1971). "Lactoferrin--specific localization in the nuclei of human polymorphonuclear neutrophilic leukocytes." *Proceedings of the Society For Experimental Biology and Medicine* **137**(4): 1311-7.

Green, M. R., C. Mycock *et al.* (1987). "Biochemical and ultrastructural processing of [125I]epidermal growth factor in rat epidermis and hair follicles: accumulation of nuclear label." *Journal of Investigative Dermatology* **88**(3): 259-65.

Greenfeder, S., S. P. Umland *et al.* (2001). "Th2 cytokines and asthma - The role of interleukin-5 in allergic eosinophilic disease." *Respir Res* **2**(2): 71-9.

Grenfell, S., N. Smithers *et al.* (1989). "Receptor-mediated endocytosis and nuclear transport of human interleukin 1 alpha." *Biochem J* **264**(3): 813-22.

Grenfell, S., N. Smithers *et al.* (1991). "Analysis of mutations in the putative nuclear localization sequence of interleukin-1 beta." *Biochem J* **280**(Pt 1): 111-6.

Gulbenkian, A. R., R. W. Egan *et al.* (1992). "Interleukin-5 modulates eosinophil accumulation in allergic guinea pig lung." *Am Rev Respir Dis* **146**(1): 263-6.

Guthridge, M. A., F. C. Stomski *et al.* (1998). "Mechanism of activation of the GM-CSF, IL-3, and IL-5 family of receptors." *Stem Cells* **16**(5): 301-13.

Hall, L. R., R. K. Mehlotra *et al.* (1998). "An essential role for interleukin-5 and eosinophils in helminth-induced airway hyperresponsiveness." *Infect Immun* **66**(9): 4425-30.

Hanover, J. A., Cohen, C. K., Willingham, M. C. & Park, M. K. (1987). "O-linked N-acetylglucosamine is attached to proteins of the nuclear pore. Evidence for cytoplasmic and nucleoplasmic glycoproteins." *J Biol Chem* **262**(20): 9887-94.

Harada, N., Y. Kikuchi *et al.* (1985). "BCGFII activity on activated B cells of a purified murine T cell-replacing factor (TRF) from a T cell hybridoma (B151K12)." *J Immunol* **134**(6): 3944-51.

Harada, S., R. M. Smith *et al.* (1993). "Inhibition of insulin-degrading enzyme increases translocation of insulin to the nucleus in H35 rat hepatoma cells: evidence of a cytosolic pathway." *Endocrinology* **132**(6): 2293-8.

Harada, S., R. M. Smith *et al.* (1994). "1,10-Phenanthroline increases nuclear accumulation of insulin in response to inhibiting insulin degradation but has a biphasic effect on insulin's ability to increase mRNA levels." *DNA and Cell Biology* **13**(5): 487-93.

Harada, S., R. M. Smith *et al.* (1999). "Mechanisms of nuclear translocation of insulin." *Cell Biochemistry and Biophysics* **31**(3): 307-19.

Hawker, J. R. J. and H. J. Granger (1994). "Nuclear accumulation of exogenous basic fibroblast growth factor in endothelial, fibroblast, and myoblast cell lines results in diverse biological responses." *In Vitro Cellular and Developmental Biology. Animal* **30A**(10): 653-63.

Henderson, J. E., N. Amizuka *et al.* (1995). "Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death." *Mol Cell Biol* **15**(8): 4064-75.

Henderson, J. E. (1997). "Nuclear targeting of secretory proteins." *Mol Cell Endocrinol* **129**(1): 1-5.

Henkel, T., U. Zabel *et al.* (1992). "Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-kappa B subunit." *Cell* **68**(6): 1121-33.

Hennekes, H., M. Peter *et al.* (1993). "Phosphorylation on protein kinase C sites inhibits nuclear import of lamin B2." *J Cell Biol* **120**(6): 1293-304.

Heyworth, C. M., T. M. Dexter *et al.* (1990). "The role of hemopoietic growth factors in self-renewal and differentiation of IL-3-dependent multipotential stem cells." *Growth Factors* **2**(2-3): 197-211.

Hetzer, M. and I. W. Mattaj (2000). "An ATP-dependent, Ran-independent mechanism for nuclear import of the U1A and U2B" spliceosome proteins." *J Cell Biol* **148**(2): 293-303.

Hitoshi, Y., N. Yamaguchi *et al.* (1990). "Distribution of IL-5 receptor-positive B cells. Expression of IL-5 receptor on Ly-1(CD5)+ B cells." *J Immunol* **144**(11): 4218-25.

Hogan, S. P., A. Koskinen *et al.* (1998). "Interleukin-5-producing CD4+ T cells play a pivotal role in aeroallergen-induced eosinophilia, bronchial hyperreactivity, and lung damage in mice." *Am J Respir Crit Care Med* **157**(1): 210-8.

Holt, S. J., P. Alexander *et al.* (1994). "Epidermal growth factor induced tyrosine phosphorylation of nuclear proteins associated with translocation of epidermal growth factor receptor into the nucleus." *Biochem Pharmacol* **47**(1): 117-26.

Hu, W. and D. A. Jans (1999). "Efficiency of importin alpha/beta-mediated nuclear localization sequence recognition and nuclear import. Differential role of NTF2." *J Biol Chem* **274**(22): 15820-7.

Hübner, S., C. Y. Xiao *et al.* (1997). "The protein kinase CK2 site (Ser111/112) enhances recognition of the simian virus 40 large T-antigen nuclear localization sequence by importin." *J Biol Chem* **272**(27): 17191-5.

Hübner, S., H. M. Smith *et al.* (1999). "Plant importin alpha binds nuclear localization sequences with high affinity and can mediate nuclear import independent of importin beta." *J Biol Chem* **274**(32): 22610-7.

Huston, M. M., J. P. Moore *et al.* (1996). "Human B cells express IL-5 receptor messenger ribonucleic acid and respond to IL-5 with enhanced IgM production after mitogenic stimulation with Moraxella catarrhalis." *J Immunol* **156**(4): 1392-401.

Ihle, J. N. (1996). "STATs: signal transducers and activators of transcription." *Cell* **84**(3): 331-4.

Ihle, J. N., T. Nosaka *et al.* (1997). "Jaks and Stats in cytokine signaling." *Stem Cells* **15**(Suppl 1): 105-11; discussion 112.

Imamura, T., K. Engleka *et al.* (1990). "Recovery of mitogenic activity of a growth factor mutant with a nuclear translocation sequence." *Science* **249**(4976): 1567-70.

Ingley, E. and I. G. Young (1991). "Characterization of a receptor for interleukin-5 on human eosinophils and the myeloid leukemia line HL-60." *Blood* **78**(2): 339-44.

Itoh, T., A. Muto *et al.* (1996). "Granulocyte-macrophage colony-stimulating factor provokes RAS activation and transcription of c-fos through different modes of signaling." *J Biol Chem* **271**(13): 7587-92.

Itoh, T., R. Liu *et al.* (1998). "Definition of the role of tyrosine residues of the common beta subunit regulating multiple signaling pathways of granulocyte-macrophage colony-stimulating factor receptor." *Mol Cell Biol* **18**(2): 742-52.

Iuchi, S. and H. Green (1997). "Nuclear localization of basonuclin in human keratinocytes and the role of phosphorylation." *Proc Natl Acad Sci U S A* **94**(15): 7948-53.

Iwama, T., H. Nagai *et al.* (1992). "Effect of murine recombinant interleukin-5 on the cell population in guinea-pig airways." *Br J Pharmacol* **105**(1): 19-22.

Jäkel, S., W. Albig *et al.* (1999). "The importin beta/importin 7 heterodimer is a functional nuclear import receptor for histone H1." *Embo J* **18**(9): 2411-23.

Jäkel, S. and D. Görlich (1998). "Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells." *Embo J* **17**(15): 4491-502.

Jans, D. A., M. J. Ackermann *et al.* (1991). "p34cdc2-mediated phosphorylation at T124 inhibits nuclear import of SV-40 T antigen proteins." *J Cell Biol* **115**(5): 1203-12.

Jans, D. A. (1994). "Nuclear signaling pathways for polypeptide ligands and their membrane receptors?" *Faseb J* **8**(11): 841-7.

Jans, D. A., T. Moll *et al.* (1995a). "Cyclin-dependent kinase site-regulated signal-dependent nuclear localization of the SW15 yeast transcription factor in mammalian cells." *J Biol Chem* **270**(29): 17064-7.

Jans, D. A. (1995b). "The regulation of protein transport to the nucleus by phosphorylation." *Biochem J* **311**(Pt 3): 705-16.

Jans, D. A. and S. Hubner (1996). "Regulation of protein transport to the nucleus: central role of phosphorylation." *Physiol Rev* **76**(3): 651-85.

Jans, D. A., L. J. Briggs *et al.* (1997a). "A functional bipartite nuclear localisation signal in the cytokine interleukin-5." *FEBS Lett* **406**(3): 315-20.

Jans, D. A., L. J. Briggs *et al.* (1997b). "The cytokine interleukin-5 (IL-5) effects cotransport of its receptor subunits to the nucleus in vitro." *FEBS Lett* **410**(2-3): 368-72.

Jans, D. A. and G. Hassan (1998). "Nuclear targeting by growth factors, cytokines, and their receptors: a role in signaling?" *Bioessays* **20**(5): 400-11.

Jans, D. A., P. Jans *et al.* (2000a). "Intranuclear binding by the HIV-1 regulatory protein VPR is dependent on cytosolic factors." *Biochem Biophys Res Commun* **270**(3): 1055-62.

Jans, D. A., C. Y. Xiao *et al.* (2000b). "Nuclear targeting signal recognition: a key control point in nuclear transport?" *Bioessays* **22**(6): 532-44.

Jenkins, Y., M. McEntee *et al.* (1998). "Characterization of HIV-1 Vpr nuclear import: analysis of signals and pathways." *J Cell Biol* **143**(4): 875-85.

Jiang, L. W. and M. Schindler (1990). "Nucleocytoplasmic transport is enhanced concomitant with nuclear accumulation of epidermal growth factor (EGF) binding activity in both 3T3-1 and EGF receptor reconstituted NR-6 fibroblasts." *J Cell Biol* **110**(3): 559-68.

Jimenez, E., G. P. Vinson *et al.* (1994). "Angiotensin II (AII)-binding sites in nuclei from rat liver: partial characterization of the mechanism of AII accumulation in nuclei." *Journal of Endocrinology* **143**(3): 449-53.

Johanson, K., E. Appelbaum *et al.* (1995). "Binding interactions of human interleukin 5 with its receptor alpha subunit. Large scale production, structural, and functional studies of Drosophila-expressed recombinant proteins." *J Biol Chem* **270**(16): 9459-71.

Johnson, G. R., T. Saeki *et al.* (1991). "Response to and expression of amphiregulin by ovarian carcinoma and normal ovarian surface epithelial cells: nuclear localization of endogenous amphiregulin." *Biochem Biophys Res Commun* **180**(2): 481-8.

Johnson, L. K., I. Vlodavsky *et al.* (1980). "Nuclear accumulation of epidermal growth factor in cultured rat pituitary cells." *Nature* **287**(5780): 340-3.

Kalderon, D., B. L. Roberts *et al.* (1984a). "A short amino acid sequence able to specify nuclear location." *Cell* **39**(3 Pt 2): 499-509.

Kalderon, D., W. D. Richardson *et al.* (1984b). "Sequence requirements for nuclear location of simian virus 40 large-T antigen." *Nature* **311**(5981): 33-8.

Katahira, J., K. Strasser *et al.* (1999). "The Mex67p-mediated nuclear mRNA export pathway is conserved from yeast to human." *Embo J* **18**(9): 2593-609.

Kay, A. B. (2001a). "Allergy and allergic diseases. First of two parts." *N Engl J Med* **344**(1): 30-7.

Kay, A. B. (2001b). "Allergy and allergic diseases. Second of two parts." *N Engl J Med* **344**(2): 109-13.

Kehlenbach, R. H., A. Dickmanns *et al.* (1999). "A role for RanBP1 in the release of CRM1 from the nuclear pore complex in a terminal step of nuclear export." *J Cell Biol* **145**(4): 645-57.

Kiefer, P., P. Acland *et al.* (1994). "Competition between nuclear localization and secretory signals determines the subcellular fate of a single CUG-initiated form of FGF3." *Embo J* **13**(17): 4126-36.

Kiefer, P. and C. Dickson (1995). "Nucleolar association of fibroblast growth factor 3 via specific sequence motifs has inhibitory effects on cell growth." *Mol Cell Biol* **15**(8): 4364-74.

Kikuchi, Y., M. Migita *et al.* (1994). "Biochemical and functional characterization of soluble form of IL-5 receptor alpha (sIL-5R alpha). Development of ELISA system for detection of sIL-5R alpha." *J Immunol Methods* **167**(1-2): 289-98.

Kimura, H. (1993). "Schwannoma-derived growth factor must be transported into the nucleus to exert its mitogenic activity." *Proc Natl Acad Sci U S A* **90**(6): 2165-9.

Kinashi, T., N. Harada *et al.* (1986). "Cloning of complementary DNA encoding T-cell replacing factor and identity with B-cell growth factor II." *Nature* **324**(6092): 70-3.

Kleinschmidt, J. A. and A. Seiter (1988). "Identification of domains involved in nuclear uptake and histone binding of protein N1 of Xenopus laevis." *Embo J* **7**(6): 1605-14.

Kobe, B. (1999). "Autoinhibition by an internal nuclear localization signal revealed by the crystal structure of mammalian importin alpha." *Nat Struct Biol* **6**(4): 388-97.

Kodama, S., M. Tsujimoto *et al.* (1993). "Role of sugar chains in the in-vitro activity of recombinant human interleukin 5." *Eur J Biochem* **211**(3): 903-8.

Koike, M. and K. Takatsu (1994). "IL-5 and its receptor: which role do they play in the immune response?" *International Archives of Allergy and Immunology* **104**(1): 1-9.

Kopf, M., F. Brombacher *et al.* (1996). "IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses." *Immunity* **4**(1): 15-24.

Kotsimbos, A. T. and Q. Hamid (1997). "IL-5 and IL-5 receptor in asthma." *Mem Inst Oswaldo Cruz* **92**(Suppl 2): 75-91.

Kubota, S., H. Siomi *et al.* (1989). "Functional similarity of HIV-I rev and HTLV-I rex proteins: identification of a new nucleolar-targeting signal in rev protein." *Biochem Biophys Res Commun* **162**(3): 963-70.

Kurup, V. P., P. S. Murali *et al.* (1997). "Anti-interleukin (IL)-4 and -IL-5 antibodies downregulate IgE and eosinophilia in mice exposed to Aspergillus antigens." *Allergy* **52**(12): 1215-21.

Kushnaryov, V. M., H. S. MacDonald *et al.* (1985). "Murine interferon-beta receptor-mediated endocytosis and nuclear membrane binding." *Proc Natl Acad Sci U S A* **82**(10): 3281-5.

Kushnaryov, V. M., H. S. MacDonald *et al.* (1986). "Internalization and transport of mouse beta-interferon into the cell nucleus." *Journal of Interferon Research* **6**(3): 241-5.

Kutay, U., E. Izaurralde *et al.* (1997). "Dominant-negative mutants of importin-beta block multiple pathways of import and export through the nuclear pore complex." *Embo J* **16**(6): 1153-63.

Lam, M. H., L. J. Briggs *et al.* (1999). "Importin beta recognizes parathyroid hormone-related protein with high affinity and mediates its nuclear import in the absence of importin alpha." *J Biol Chem* **274**(11): 7391-8.

Lam, M. H., R. J. Thomas *et al.* (2000). "Nuclear and nucleolar localization of parathyroid hormone-related protein." *Immunol Cell Biol* **78**(4): 395-402.

Lam, M. H., W. Hu *et al.* (2001). "Molecular dissection of the importin beta1-recognized nuclear targeting signal of parathyroid hormone-related protein." *Biochem Biophys Res Commun* **282**(2): 629-34.

Lam, M. H., R. J. Thomas *et al.* (2002). "Nuclear transport of parathyroid hormone (PTH)-related protein is dependent on microtubules." *Mol Endocrinol* **16**(2): 390-401.

Lanford, R. E. and J. S. Butel (1984). "Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen." *Cell* **37**(3): 801-13.

Lanford, R. E., C. M. Feldherr *et al.* (1990). "Comparison of diverse transport signals in synthetic peptide-induced nuclear transport." *Exp Cell Res* **186**(1): 32-8.

Larkin, J., 3rd, H. M. Johnson *et al.* (2000). "Differential nuclear localization of the IFNGR-1 and IFNGR-2 subunits of the IFN-gamma receptor complex following activation by IFN-gamma." *J Interferon Cytokine Res* **20**(6): 565-76.

Lee, B. A., D. W. Maher *et al.* (1987). "Identification of a signal for nuclear targeting in platelet-derived-growth-factor-related molecules." *Molecular and Cellular Biology* **7**(10): 3527-37.

Leonard, W. J. and J. X. Lin (2000). "Cytokine receptor signaling pathways." *J Allergy Clin Immunol* **105**(5): 877-88.

Letuve, S., A. Druilhe *et al.* (2001). "Involvement of caspases and of mitochondria in Fas ligation-induced eosinophil apoptosis: modulation by interleukin-5 and interferon-gamma." *J Leukoc Biol* **70**(5): 767-75.

Leverrier, Y., J. Thomas *et al.* (1999). "Role of PI3-kinase in Bcl-X induction and apoptosis inhibition mediated by IL-3 or IGF-1 in Baf-3 cells." *Cell Death Differ* **6**(3): 290-6.

Li, J., R. Cook *et al.* (1996). "Single chain human interleukin 5 and its asymmetric mutagenesis for mapping receptor binding sites." *J Biol Chem* **271**(4): 1817-20.

Li, W. and G. Keller (2000). "VEGF nuclear accumulation correlates with phenotypical changes in endothelial cells." *J Cell Sci* **113**(Pt 9): 1525-34.

Li, X. and L. D. Etkin (1993). "Xenopus nuclear factor 7 (xnf7) possesses an NLS that functions efficiently in both oocytes and embryos." *J Cell Sci* **105**(Pt 2): 389-95.

Lin, S. Y., K. Makino *et al.* (2001). "Nuclear localization of EGF receptor and its potential new role as a transcription factor." *Nat Cell Biol* **3**(9): 802-8.

Lin, Y. Z., S. Y. Yao *et al.* (1996). "Role of the nuclear localization sequence in fibroblast growth factor-1-stimulated mitogenic pathways." *J Biol Chem* **271**(10): 5305-8.

Lixin, R., A. Efthymiadis *et al.* (2001). "Novel properties of the nucleolar targeting signal of human angiogenin." *Biochem Biophys Res Commun* **284**(1): 185-93.

Lobie, P. E., R. Barnard *et al.* (1991). "The nuclear growth hormone receptor binding protein. Antigenic and physicochemical characterization." *J Biol Chem* **266**(33): 22645-52.

Lobie, P. E., W. Breipohl *et al.* (1990). "Cellular localization of the growth hormone receptor/binding protein in the male and female reproductive systems." *Endocrinology* **126**(4): 2214-21.

Lobie, P. E., H. Mertani *et al.* (1994a). "Receptor-mediated nuclear translocation of growth hormone." *J Biol Chem* **269**(33): 21330-9.

Lobie, P. E., T. J. Wood *et al.* (1994b). "Nuclear translocation and anchorage of the growth hormone receptor." *Journal of Biological Chemistry* **269**(50): 31735-46.

Lopez, A. F., M. A. Vadas *et al.* (1991). "Interleukin-5, interleukin-3, and granulocyte-macrophage colony-stimulating factor cross-compete for binding to cell surface receptors on human eosinophils." *J Biol Chem* **266**(36): 24741-7.

Lu, D., H. Yang *et al.* (1998a). "Involvement of p62 nucleoporin in angiotensin II-induced nuclear translocation of STAT3 in brain neurons." *Journal of Neuroscience* **18**(4): 1329-36.

Lu, D., H. Yang *et al.* (1998b). "Angiotensin II-induced nuclear targeting of the angiotensin type 1 (AT1) receptor in brain neurons." *Endocrinology* **139**(1): 365-75.

MacDonald, H. S., V. M. Kushnaryov *et al.* (1986). "Transport of gamma-interferon into the cell nucleus may be mediated by nuclear membrane receptors." *Biochem Biophys Res Commun* **138**(1): 254-60.

Mahanty, S., J. S. Abrams *et al.* (1992). "Parallel regulation of IL-4 and IL-5 in human helminth infections." *J Immunol* **148**(11): 3567-71.

Mahanty, S. and T. B. Nutman (1993). "The biology of interleukin-5 and its receptor." *Cancer Investigation* **11**(5): 624-34.

Maher, D. W., B. A. Lee *et al.* (1989). "The alternatively spliced exon of the platelet-derived growth factor A chain encodes a nuclear targeting signal." *Molecular and Cellular Biology* **9**(5): 2251-3.

Maher, P. A. (1996). "Nuclear Translocation of fibroblast growth factor (FGF) receptors in response to FGF-2." *Journal of Cell Biology* **134**(2): 529-36.

Mandler, R., C. C. Chu *et al.* (1993). "Interleukin 5 induces S mu-S gamma 1 DNA rearrangement in B cells activated with dextran-anti-IgD antibodies and interleukin 4: a three component model for Ig class switching." *J Exp Med* **178**(5): 1577-86.

Manrow, R. E., A. R. Sburlati *et al.* (1991). "Nuclear targeting of prothymosin alpha." *J Biol Chem* **266**(6): 3916-24.

Martinez-Moczygemba, M. and D. P. Huston (2001). "Proteasomal regulation of betac signaling reveals a novel mechanism for cytokine receptor heterotypic desensitization." *J Clin Invest* **108**(12): 1797-806.

Mathey-Prevot, B. and N. Perrimon (1998). "Mammalian and Drosophila blood: JAK of all trades?" *Cell* **92**(6): 697-700.

Matsuguchi, T., R. C. Inhorn *et al.* (1995). "Tyrosine phosphorylation of p95Vav in myeloid cells is regulated by GM-CSF, IL-3 and steel factor and is constitutively increased by p210BCR/ABL." *Embo J* **14**(2): 257-65.

Matsuguchi, T., Y. Zhao *et al.* (1997). "The cytoplasmic domain of granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor alpha subunit is essential for both GM-CSF-mediated growth and differentiation." *J Biol Chem* **272**(28): 17450-9.

McKenzie, A. N., B. Ely *et al.* (1991). "Mutated interleukin-5 monomers are biologically inactive." *Mol Immunol* **28**(1-2): 155-8.

McKenzie, D. T., H. I. Filutowicz *et al.* (1987). "Purification and partial sequence analysis of murine B cell growth factor II (interleukin 5)." *J Immunol* **139**(8): 2661-8.

McMillan, D. N., N. M. Kernohan *et al.* (1995). "Interleukin 2 receptor expression and interleukin 2 localisation in human solid tumor cells in situ and in vitro: evidence for a direct role in the regulation of tumour cell proliferation." *Int J Cancer* **60**(6): 766-72.

Meeusen, E. N. and A. Balic (2000). "Do eosinophils have a role in the killing of helminth parasites?" *Parasitol Today* **16**(3): 95-101.

Melchior, F., B. Paschal *et al.* (1993). "Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor." *J Cell Biol* **123**(6 Pt 2): 1649-59.

Mertani, H. C., G. Morel *et al.* (1999). "Cytoplasmic and nuclear cytokine receptor complexes." *Vitamins and Hormones* **57**: 79-121.

Michael, W. M., M. Choi *et al.* (1995a). "A nuclear export signal in hnRNP A1: a signal-mediated, temperature- dependent nuclear protein export pathway." *Cell* **83**(3): 415-22.

Michael, W. M., H. Siomi *et al.* (1995b). "Signal sequences that target nuclear import and nuclear export of pre- mRNA-binding proteins." *Cold Spring Harb Symp Quant Biol* **60**: 663-8.

Migita, M., N. Yamaguchi *et al.* (1991). "Characterization of the human IL-5 receptors on eosinophils." *Cell Immunol* **133**(2): 484-97.

Milburn, M. V., A. M. Hassell *et al.* (1993). "A novel dimer configuration revealed by the crystal structure at 2.4 A resolution of human interleukin-5." *Nature* **363**(6425): 172-6.

Minamitake, Y., S. Kodama *et al.* (1990). "Structure of recombinant human interleukin 5 produced by Chinese hamster ovary cells." *J Biochem (Tokyo)* **107**(2): 292-7.

Mingot, J. M., S. Kostka *et al.* (2001). "Importin 13: a novel mediator of nuclear import and export." *Embo J* **20**(14): 3685-94.

Mire-Sluis, A., L. A. Page *et al.* (1995). "Evidence for a signaling role for the alpha chains of granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-5 receptors: divergent signaling pathways between GM-CSF/IL-3 and IL-5." *Blood* **86**(7): 2679-88.

Mita, S., A. Tominaga *et al.* (1989a). "Characterization of high-affinity receptors for interleukin 5 on interleukin 5-dependent cell lines." *Proc Natl Acad Sci U S A* **86**(7): 2311-5.

Mita, S., Y. Hosoya *et al.* (1989b). "Rapid methods for purification of human recombinant interleukin-5 (IL-5) using the anti-murine IL-5 antibody-coupled immunoaffinity column." *J Immunol Methods* **125**(1-2): 233-41.

Mita, S., S. Takaki *et al.* (1991). "Molecular characterization of the beta chain of the murine interleukin 5 receptor." *Int Immunol* **3**(7): 665-72.

Miyajima, A., A. L. Mui *et al.* (1993). "Receptors for granulocyte-macrophage colony-stimulating factor, interleukin-3, and interleukin-5." *blood* **82**(7): 1960-74.

Mo, Y. Y., C. Wang *et al.* (2000). "A novel nuclear localization signal in human DNA topoisomerase I." *J Biol Chem* **275**(52): 41107-13.

Modrell, B., V. L. McDonald *et al.* (1992). "The interaction of amphiregulin with nuclei and putative nuclear localization sequence binding proteins." *Growth Factors* **7**(4): 305-14.

Moll, T., G. Tebb *et al.* (1991). "The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the S. cerevisiae transcription factor SWI5." *Cell* **66**(4): 743-58.

Moore, M. S. and G. Blobel (1994). "A G protein involved in nucleocytoplasmic transport: the role of Ran." *Trends Biochem Sci* **19**(5): 211-6.

Morel, G. (1994). "Internalization and nuclear localization of peptide hormones." *Biochem Pharmacol* **47**(1): 63-76.

Moreland, R. B., G. L. Langevin *et al.* (1987). "Amino acid sequences that determine the nuclear localization of yeast histone 2B." *Mol Cell Biol* **7**(11): 4048-57.

Moroianu, J. and J. F. Riordan (1994a). "Nuclear translocation of angiogenic proteins in endothelial cells: an essential step in angiogenesis." *Biochemistry* **33**(42): 12535-9.

Moroianu, J. and J. F. Riordan (1994b). "Nuclear translocation of angiogenin in proliferating endothelial cells is essential to its angiogenic activity." *Proc Natl Acad Sci U S A* **91**(5): 1677-81.

Mui, A. L., H. Wakao *et al.* (1995). "Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs." *Embo J* **14**(6): 1166-75.

Murali, P. S., A. Kumar *et al.* (1993). "Aspergillus fumigatus antigen induced eosinophilia in mice is abrogated by anti-IL-5 antibody." *J Leukoc Biol* **53**(3): 264-7.

Murata, Y., S. Takaki *et al.* (1992). "Molecular cloning and expression of the human interleukin 5 receptor." *J Exp Med* **175**(2): 341-51.

Murawaki, Y., R. Storkenmaier *et al.* (1990). "Effects of chloroquine and hepatic stimulator substance on cellular accumulation and nuclear binding of 125I-epidermal growth factor in primary culture of adult rat hepatocytes." *Res Commun Chem Pathol Pharmacol* **69**(1): 3-15.

Nagai, H., S. Yamaguchi *et al.* (1993). "Priming with murine recombinant interleukin-5 resulted in the augmentation of PAF-induced airway hyperresponsiveness to histamine in guinea pigs." *Life Sci* **52**(17): L147-51.

Nagai, H., S. Yamaguchi *et al.* (1996a). "The role of interleukin-5 (IL-5) in allergic airway hyperresponsiveness in mice." *Ann N Y Acad Sci* **796**: 91-6.

Nagai, H., S. Yamaguchi *et al.* (1996b). "Role of mast cells, eosinophils and IL-5 in the development of airway hyperresponsiveness in sensitized mice." *Clin Exp Allergy* **26**(6): 642-7.

Nakanishi, Y., K. Kihara *et al.* (1992). "Direct effect of basic fibroblast growth factor on gene transcription in a cell-free system." *Proc Natl Acad Sci U S A* **89**(12): 5216-20.

Nakielny, S., S. Shaikh *et al.* (1999). "Nup153 is an M9-containing mobile nucleoporin with a novel Ran-binding domain." *Embo J* **18**(7): 1982-95.

Naora, H., B. H. van Leeuwen *et al.* (1994b). "Functional role and signal-induced modulation of proteins recognizing the conserved TCATTT-containing promoter elements in the murine IL-5 and GM-CSF genes in T lymphocytes." *J Immunol* **153**(8): 3466-75.

Naora, H. and I. G. Young (1994a). "Mechanisms regulating the mRNA levels of interleukin-5 and two other coordinately expressed lymphokines in the murine T lymphoma EL4.23." *Blood* **83**(12): 3620-8.

Nehrbass, U. and G. Blobel (1996). "Role of the nuclear transport factor p10 in nuclear import." *Science* **272**(5258): 120-2.

Newmeyer, D. D. and D. J. Forbes (1988). "Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation." *Cell* **52**(5): 641-53.

Nguyen, M. T. and A. C. Karaplis (1998). "The nucleus: a target site for parathyroid hormone-related peptide (PTHrP) action." *J Cell Biochem* **70**(2): 193-9.

Nicola, N. (1994). Guidebook to Cytokines and Their Receptors. New York, Oxford University Press, Sambrook and Tooze Publications: 171-177.

Nigg, E. A. (1997). "Nucleocytoplasmic transport: signals, mechanisms and regulation." *Nature* **386**(6627): 779-87.

Ogata, N., T. Kouro *et al.* (1998). "JAK2 and JAK1 constitutively associate with an interleukin-5 (IL-5) receptor alpha and betac subunit, respectively, and are activated upon IL-5 stimulation." *Blood* **91**(7): 2264-71.

Okuda, K., L. Smith *et al.* (1997). "Signaling functions of the tyrosine residues in the betac chain of the granulocyte-macrophage colony-stimulating factor receptor." *Blood* **90**(12): 4759-66.

Paine, P. L., L. C. Moore *et al.* (1975). "Nuclear envelope permeability." *Nature* **254**(5496): 109-14.

Palmeri, D. and M. H. Malim (1999). "Importin beta can mediate the nuclear import of an arginine-rich nuclear localization signal in the absence of importin alpha." *Mol Cell Biol* **19**(2): 1218-25.

Palvimo, J. and A. Linnala-Kankkunen (1990). "Identification of a low-Mr acidic nuclear protein as prothymosin alpha." *FEBS Lett* **277**(1-2): 257-60.

Park, M. K., M. D'Onofrio *et al.* (1987). "A monoclonal antibody against a family of nuclear pore proteins (nucleoporins): O-linked N-acetylglucosamine is part of the immunodeterminant." *Proc Natl Acad Sci U S A* **84**(18): 6462-6.

Paul, C. C., M. Tolbert *et al.* (1993). "Cooperative effects of interleukin-3 (IL-3), IL-5, and granulocyte-macrophage colony-stimulating factor: a new myeloid cell line inducible to eosinophils." *Blood* **81**(5): 1193-9.

Pazdrak, K., S. Stafford *et al.* (1995a). "The activation of the Jak-STAT 1 signaling pathway by IL-5 in eosinophils." *J Immunol* **155**(1): 397-402.

Pazdrak, K., D. Schreiber *et al.* (1995b). "The intracellular signal transduction mechanism of interleukin 5 in eosinophils: the involvement of lyn tyrosine kinase and the Ras-Raf-1-MEK-microtubule-associated protein kinase pathway." *J Exp Med* **181**(5): 1827-34.

Pazdrak, K., B. Olszewska-Pazdrak *et al.* (1998). "Lyn, Jak2, and Raf-1 kinases are critical for the antiapoptotic effect of interleukin 5, whereas only Raf-1 kinase is essential for eosinophil activation and degranulation." *J Exp Med* **188**(3): 421-9.

Peculis, B. A. and J. G. Gall (1992). "Localization of the nucleolar protein NO38 in amphibian oocytes." *J Cell Biol* **116**(1): 1-14.

Pederson, T. (1998). "Growth factors in the nucleolus?" *J Cell Biol* **143**(2): 279-81.

Peng, H., J. Moffett *et al.* (2001). "Novel nuclear signaling pathway mediates activation of fibroblast growth factor-2 gene by type 1 and type 2 angiotensin II receptors." *Mol Biol Cell* **12**(2): 449-62.

Perbal, B. (1999). "Nuclear localisation of NOVH protein: a potential role for NOV in the regulation of gene expression." *Mol Pathol* **52**(2): 84-91.

Picard, D., V. Kumar *et al.* (1990). "Signal transduction by steroid hormones: nuclear localization is differentially regulated in estrogen and glucocorticoid receptors." *Cell Regul* **1**(3): 291-9.

Podlecki, D. A., R. M. Smith *et al.* (1987). "Nuclear translocation of the insulin receptor. A possible mediator of insulin's long term effects." *J Biol Chem* **262**(7): 3362-8.

Pollard, V. W., W. M. Michael *et al.* (1996). "A novel receptor-mediated nuclear protein import pathway." *Cell* **86**(6): 985-94.

Powers, C. J., S. W. McLeskey *et al.* (2000). "Fibroblast growth factors, their receptors and signaling." *Endocr Relat Cancer* **7**(3): 165-97.

Powers, M. A., D. J. Forbes *et al.* (1997). "The vertebrate GLFG nucleoporin, Nup98, is an essential component of multiple RNA export pathways." *J Cell Biol* **136**(2): 241-50.

Prochiantz, A. and L. Theodore (1995). "Nuclear/growth factors." *Bioessays* **17**(1): 39-44.

Proudfoot, A. E., J. G. Davies *et al.* (1991). "Human interleukin-5 expressed in Escherichia coli: assignment of the disulfide bridges of the purified unglycosylated protein." *FEBS Lett* **283**(1): 61-4.

Prudovsky, I. and Z. X. Savion N, Friesel R, Xu J, Hou J, McKeehan WL, Maciag T (1994). "Intact and functional fibroblast growth factor (FGF) receptor-1 trafficks near the nucleus in response to FGF-1." *J Biol Chem* **269**(50): 31720-4.

Quarto, N., F. P. Finger *et al.* (1991). "The NH2-terminal extension of high molecular weight bFGF is a nuclear targeting signal." *Journal of Cellular Physiology* **147**(2): 311-8.

Quelle, F. W., N. Sato *et al.* (1994). "JAK2 associates with the beta c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region." *Mol Cell Biol* **14**(7): 4335-41.

Radu, A., G. Blobel *et al.* (1995a). "Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins." *Proc Natl Acad Sci U S A* **92**(5): 1769-73.

Radu, A., M. S. Moore *et al.* (1995b). "The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex." *Cell* **81**(2): 215-22.

Rakowicz-Szulczynska, E. M. and H. Koprowski (1986a). "Identification of NGF receptor in chromatin of melanoma cells using monoclonal antibody to cell surface NGF receptor." *Biochem Biophys Res Commun* **140**(1): 174-80.

Rakowicz-Szulczynska, E. M., U. Rodeck *et al.* (1986b). "Chromatin binding of epidermal growth factor, nerve growth factor, and platelet-derived growth factor in cells bearing the appropriate surface receptors." *Proc Natl Acad Sci U S A* **83**(11): 3728-32.

Rakowicz-Szulczynska, E. M., D. Otwiaska *et al.* (1989). "Epidermal growth factor (EGF) and monoclonal antibody to cell surface EGF receptor bind to the same chromatin receptor." *Arch Biochem Biophys* **268**(2): 456-64.

Rao, Y. P., D. J. Buckley *et al.* (1995a). "Nuclear translocation of prolactin: collaboration of tyrosine kinase and protein kinase C activation in rat Nb2 node lymphoma cells." *J Cell Physiol* **163**(2): 266-76.

Rao, Y. P., D. J. Buckley *et al.* (1995b). "The nuclear prolactin receptor: a 62-kDa chromatin-associated protein in rat Nb2 lymphoma cells." *Arch Biochem Biophys* **322**(2): 506-15.

Raper, S. E., S. J. Burwen *et al.* (1987). "Translocation of epidermal growth factor to the hepatocyte nucleus during rat liver regeneration." *gastroenterology* **92**(5 Pt 1): 1243-50.

Re, R. N., D. L. Vizard *et al.* (1984a). "Angiotensin II receptors in chromatin." *J Hypertens Suppl* **2**(3): S271-3.

Re, R. N., D. L. Vizard *et al.* (1984b). "Angiotensin II receptors in chromatin fragments generated by micrococcal nuclease." *Biochem Biophys Res Commun* **119**(1): 220-7.

Reilly, J. F. and P. A. Maher (2001). "Importin beta-mediated nuclear import of fibroblast growth factor receptor: role in cell proliferation." *J Cell Biol* **152**(6): 1307-12.

Rexach, M. and G. Blobel (1995). "Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins." *Cell* **83**(5): 683-92.

Rezvani, I., V. T. Maddaiah *et al.* (1973). "Kinetics of uptake and subcellular distribution of human growth hormone in liver slices." *Biochem Med* **7**(3): 432-40.

Richardson, W. D., B. L. Roberts *et al.* (1986). "Nuclear location signals in polyoma virus large-T." *Cell* **44**(1): 77-85.

Richardson, W. D., A. D. Mills *et al.* (1988). "Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores." *Cell* **52**(5): 655-64.

Rifkin, D. B., N. Quarto *et al.* (1991). "New observations on the intracellular localization and release of bFGF." *Annals of the New York Academy of Sciences* **638**: 204-6.

Rifkin, D. B., D. Moscatelli *et al.* (1994). "Studies on FGF-2: nuclear localization and function of high molecular weight forms and receptor binding in the absence of heparin." *Molecular Reproduction and Development* **39**(1): 102-4; discussion 104-5.

Rihs, H. P., D. A. Jans *et al.* (1991). "The rate of nuclear cytoplasmic protein transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV40 T-antigen." *Embo J* **10**(3): 633-9.

Robbins, J., S. M. Dilworth *et al.* (1991). "Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence." *Cell* **64**(3): 615-23.

Robertson, A. L. J. and P. A. Khairallah (1971). "Angiotensin II: rapid localization in nuclei of smooth and cardiac muscle." *Science* **172**(988): 1138-9.

Rohrer, H., T. Schafer *et al.* (1982). "Internalization of nerve growth factor by pheochromocytoma PC12 cells: absence of transfer to the nucleus." *journal of neuroscience* **2**(6): 687-97.

Rosa Santos, S. C., S. Dumon *et al.* (2000). "Cooperation between STAT5 and phosphatidylinositol 3-kinase in the IL-3-dependent survival of a bone marrow derived cell line." *Oncogene* **19**(9): 1164-72.

Rossi, F., M. McNagny *et al.* (1996). "Lineage commitment of transformed haematopoietic progenitors is determined by the level of PKC activity." *Embo J* **15**(8): 1894-901.

Rout, M. P. and G. Blobel (1993). "Isolation of the yeast nuclear pore complex." *J Cell Biol* **123**(4): 771-83.

Rout, M. P., J. D. Aitchison *et al.* (2000). "The yeast nuclear pore complex: composition, architecture, and transport mechanism." *J Cell Biol* **148**(4): 635-51.

Rout, M. P. and J. D. Aitchison (2001). "The nuclear pore complex as a transport machine." *J Biol Chem* **276**(20): 16593-6.

Rubtsov, Y. P., A. S. Zolotukhin *et al.* (1997). "Mutational analysis of human prothymosin alpha reveals a bipartite nuclear localization signal." *FEBS Lett* **413**(1): 135-41.

Ryan, K. J. and S. R. Wente (2000). "The nuclear pore complex: a protein machine bridging the nucleus and cytoplasm." *Curr Opin Cell Biol* **12**(3): 361-71.

Rycyzyn, M. A., S. C. Reilly *et al.* (2000). "Role of cyclophilin B in prolactin signal transduction and nuclear retrotranslocation." *Mol Endocrinol* **14**(8): 1175-86.

Rycyzyn, M. A. and C. V. Clevenger (2002). "The intranuclear prolactin/cyclophilin B complex as a transcriptional inducer." *Proc Natl Acad Sci U S A* **99**(10): 6790-5.

Sanderson, C. J., D. J. Warren *et al.* (1985). "Identification of a lymphokine that stimulates eosinophil differentiation in vitro. Its relationship to interleukin 3, and functional properties of eosinophils produced in cultures." *J Exp Med* **162**(1): 60-74.

Sanderson, C. J., H. D. Campbell *et al.* (1988). "Molecular and cellular biology of eosinophil differentiation factor (interleukin-5) and its effects on human and mouse B cells." *Immunol Rev* **102**: 29-50.

Sanderson, C. J. (1990a). "Eosinophil differentiation factor (interleukin-5)." *Immunol Ser* **49**: 231-56.

Sanderson, C. J. (1990b). "The biological role of interleukin 5." *Int J Cell Cloning* **8**(Suppl 1): 147-53; discussion 153-4.

Sanderson, C. J. (1992). "Interleukin-5, eosinophils, and disease." *Blood* **79**(12): 3101-9.

Santos, S. C., V. Lacronique *et al.* (2001). "Constitutively active STAT5 variants induce growth and survival of hematopoietic cells through a PI 3-kinase/Akt dependent pathway." *Oncogene* **20**(17): 2080-90.

Sasaki, O., H. Sugaya *et al.* (1993). "Ablation of eosinophils with anti-IL-5 antibody enhances the survival of intracranial worms of Angiostrongylus cantonensis in the mouse." *Parasite Immunol* **15**(6): 349-54.

Sato, N., C. Caux *et al.* (1993a). "Expression and factor-dependent modulation of the interleukin-3 receptor subunits on human hematopoietic cells." *Blood* **82**(3): 752-61.

Sato, N., K. Sakamaki *et al.* (1993b). "Signal transduction by the high-affinity GM-CSF receptor: two distinct cytoplasmic regions of the common beta subunit responsible for different signaling." *Embo J* **12**(11): 4181-9.

Sato, S., T. Katagiri *et al.* (1994). "IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton's tyrosine and Janus 2 kinases." *J Exp Med* **180**(6): 2101-11.

Satoh, T., M. Nakafuku *et al.* (1991). "Involvement of ras p21 protein in signal-transduction pathways from interleukin 2, interleukin 3, and granulocyte/macrophage colony-stimulating factor, but not from interleukin 4." *Proc Natl Acad Sci U S A* **88**(8): 3314-8.

Savion, N., I. Vlodavsky *et al.* (1981). "Nuclear accumulation of epidermal growth factor in cultured bovine corneal endothelial and granulosa cells." *J Biol Chem* **256**(3): 1149-54.

Schedlich, L. J., T. F. Young *et al.* (1998). "Insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 share a common nuclear transport pathway in T47D human breast carcinoma cells." *J Biol Chem* **273**(29): 18347-52.

Schedlich, L. J., S. L. Le Page *et al.* (2000). "Nuclear import of insulin-like growth factor-binding protein-3 and -5 is mediated by the importin beta subunit." *J Biol Chem* **275**(31): 23462-70.

Schimpl, A. and E. Wecker (1972). "Replacement of T-cell function by a T-cell product." *Nat New Biol* **237**(70): 15-7.

Schmalz, D., F. Hucho *et al.* (1998). "Nuclear import of protein kinase C occurs by a mechanism distinct from the mechanism used by proteins with a classical nuclear localization signal." *J Cell Sci* **111**(Pt 13): 1823-30.

Schmidt-Zachmann, M. S. and E. A. Nigg (1993). "Protein localization to the nucleolus: a search for targeting domains in nucleolin." *J Cell Sci* **105**(Pt 3): 799-806.

Schreiber, V., M. Molinete *et al.* (1992). "The human poly(ADP-ribose) polymerase nuclear localization signal is a bipartite element functionally separate from DNA binding and catalytic activity." *Embo J* **11**(9): 3263-9.

Sekimoto, T., N. Imamoto *et al.* (1997). "Extracellular signal-dependent nuclear import of Stat1 is mediated by nuclear pore-targeting complex formation with NPI-1, but not Rch1." *Embo J* **16**(23): 7067-77.

Shah, N., S. Zhang *et al.* (1995). "Electron microscopic visualization of insulin translocation into the cytoplasm and nuclei of intact H35 hepatoma cells using covalently linked Nanogold-insulin." *Endocrinology* **136**(7): 2825-35.

Shah, S. and D. J. Forbes (1998). "Separate nuclear import pathways converge on the nucleoporin Nup153 and can be dissected with dominant-negative inhibitors." *Curr Biol* **8**(25): 1376-86.

Shakulov, V. R., I. A. Vorobjev *et al.* (2000). "Interaction of yeast importin alpha with the NLS of prothymosin alpha is insufficient to trigger nuclear uptake of cargos." *Biochem Biophys Res Commun* **274**(2): 548-52.

Sherman, M. P., C. M. de Noronha *et al.* (2001). "Nucleocytoplasmic shuttling by human immunodeficiency virus type 1 Vpr." *J Virol* **75**(3): 1522-32.

Shiurba, R. A., N. Jing *et al.* (1991). "Nuclear translocation of fibroblast growth factor during Xenopus mesoderm induction." *Development* **113**(2): 487-93.

Sibon, O. C., B. M. Humbel *et al.* (1994). "Ultrastructural localization of epidermal growth factor (EGF)-receptor transcripts in the cell nucleus using pre-embedding in situ hybridization in combination with ultra-small gold probes and silver enhancement." *Histochemistry* **101**(3): 223-32.

Silver, P. A., L. P. Keegan *et al.* (1984). "Amino terminus of the yeast GAL4 gene product is sufficient for nuclear localization." *Proc Natl Acad Sci U S A* **81**(19): 5951-5.

Siomi, H., H. Shida *et al.* (1990). "Effects of a highly basic region of human immunodeficiency virus Tat protein on nucleolar localization." *J Virol* **64**(4): 1803-7.

Siomi, H. and G. Dreyfuss (1995). "A nuclear localization domain in the hnRNP A1 protein." *J Cell Biol* **129**(3): 551-60.

Siomi, M. C., P. S. Eder *et al.* (1997). "Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins." *J Cell Biol* **138**(6): 1181-92.

Sklar, L. A., D. A. Finney *et al.* (1984). "The dynamics of ligand-receptor interactions. Real-time analyses of association, dissociation, and internalization of an N-formyl peptide and its receptors on the human neutrophil." *J Biol Chem* **259**(9): 5661-9.

Smith, A., D. Metcalf *et al.* (1997). "Cytoplasmic domains of the common beta-chain of the GM-CSF/IL-3/IL-5 receptors that are required for inducing differentiation or clonal suppression in myeloid leukaemic cell lines." *Embo J* **16**(3): 451-64.

Smith, R. M. and L. Jarett (1987). "Ultrastructural evidence for the accumulation of insulin in nuclei of intact 3T3-L1 adipocytes by an insulin-receptor mediated process." *Proc Natl Acad Sci U S A* **84**(2): 459-63.

Smith, R. M. and L. Jarett (1990). "Partial characterization of mechanism of insulin accumulation in H35 hepatoma cell nuclei." *Diabetes* **396**(6): 683-9.

Solari, R., N. Smithers *et al.* (1994). "Receptor mediated endocytosis and intracellular fate of interleukin 1." *Biochemical Pharmacology* **47**(1): 93-101.

Stachowiak, M. K., P. A. Maher *et al.* (1996a). "Nuclear localization of functional FGF receptor 1 in human astrocytes suggests a novel mechanism for growth factor action." *Brain Research. Molecular Brain Research* **38**(1): 161-5.

Stachowiak, M. K., P. A. Maher *et al.* (1996b). "Nuclear accumulation of fibroblast growth factor receptors is regulated by multiple signals in adrenal medullary cells." *Molecular Biology of the Cell* **7**(8): 1299-317.

Stachowiak, M. K., J. Moffett *et al.* (1997b). "Growth factor regulation of cell growth and proliferation in the nervous system. A new intracrine nuclear mechanism." *Mol Neurobiol* **15**(3): 257-83.

Stachowiak, E. K., P. A. Maher *et al.* (1997a). "Nuclear accumulation of fibroblast growth factor receptors in human glial cells-association with cell proliferation." *Oncogene* **14**(18): 2201-11.

Stafford, S., C. Lowell *et al.* (2002). "Lyn tyrosine kinase is important for IL-5-stimulated eosinophil differentiation." *J Immunol* **168**(4): 1978-83.

Stein, O. and J. Gross (1959). *Endocrinology* **65**: 707-716.

Stochaj, U. and P. Silver (1992). "Nucleocytoplasmic traffic of proteins." *Eur J Cell Biol* **59**(1): 1-11.

Stoffler, D., B. Fahrenkrog *et al.* (1999). "The nuclear pore complex: from molecular architecture to functional dynamics." *Curr Opin Cell Biol* **11**(3): 391-401.

Stomski, F. C., M. Dottore *et al.* (1999). "Identification of a 14-3-3 binding sequence in the common beta chain of the granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-5 receptors that is serine-phosphorylated by GM-CSF." *Blood* **94**(6): 1933-42.

Subbramanian, R. A., X. J. Yao *et al.* (1998). "Human immunodeficiency virus type 1 Vpr localization: nuclear transport of a viral protein modulated by a putative amphipathic helical structure and its relevance to biological activity." *J Mol Biol* **278**(1): 13-30.

Subramaniam, P. S., M. G. Mujtaba *et al.* (1999). "The carboxyl terminus of interferon-gamma contains a functional polybasic nuclear localization sequence." *Journal of Biological Chemistry* **274**(1): 403-7.

Subramaniam, P. S., J. r. Larkin *et al.* (2000). "The COOH-terminal nuclear localization sequence of interferon gamma regulates STAT1 alpha nuclear translocation at an intracellular site." *Journal of Cell Science* **113 (Pt 15)**: 2771-81.

Subramaniam, P. S., M. M. Green *et al.* (2001). "Nuclear Translocation of IFN-gamma Is an Intrinsic Requirement for Its Biologic Activity and Can Be Driven by a Heterologous Nuclear Localization Sequence." *J Interferon Cytokine Res* **21**(11): 951-959.

Sun, Z., D. A. Yergeau *et al.* (1996). "Interleukin-5 receptor alpha subunit gene regulation in human eosinophil development: identification of a unique cis-element that acts lie an enhancer in regulating activity of the IL-5R alpha promoter." *Curr Top Microbiol Immunol* **211**: 173-87.

Tagari, P., E. I. Pecheur *et al.* (1993). "Activation of human eosinophils and differentiated HL-60 cells by interleukin-5." *Int Arch Allergy Immunol* **101**(3): 227-33.

Takahashi, K., K. Suzuki *et al.* (1997). "Induction of tyrosine phosphorylation and association of beta-catenin with EGF receptor upon tryptic digestion of quiescent cells at confluence." *Oncogene* **15**(1): 71-8.

Takaki, S., S. Mita *et al.* (1991). "Identification of the second subunit of the murine interleukin-5 receptor: interleukin-3 receptor-like protein, AIC2B is a component of the high affinity interleukin-5 receptor." *Embo J* **10**(10): 2833-8.

Takaki, S., Y. Murata *et al.* (1993). "Reconstitution of the functional receptors for murine and human interleukin 5." *J Exp Med* **177**(6): 1523-9.

Takaki, S., A. Tominaga *et al.* (1990). "Molecular cloning and expression of the murine interleukin-5 receptor." *Embo J* **9**(13): 4367-74.

Takatsu, K., S. Takaki *et al.* (1994). "Interleukin-5 and its receptor system: implications in the immune system and inflammation." *Adv Immunol* **57**: 145-90.

Tan, P. B. and S. K. Kim (1999). "Signaling specificity: the RTK/RAS/MAP kinase pathway in metazoans." *Trends Genet* **15**(4): 145-9.

Tang, S. S., H. Rogg *et al.* (1992). "Characterization of nuclear angiotensin-II-binding sites in rat liver and comparison with plasma membrane receptors." *Endocrinology* **131**(1): 374-80.

Tavernier, J., R. Devos *et al.* (1989). "Expression of human and murine interleukin-5 in eukaryotic systems." *DNA* **8**(7): 491-501.

Tavernier, J., R. Devos *et al.* (1991). "A human high affinity interleukin-5 receptor (IL5R) is composed of an IL5-specific alpha chain and a beta chain shared with the receptor for GM-CSF." *Cell* **66**(6): 1175-84.

Tavernier, J., S. Cornelis *et al.* (1995). "Structure/function analysis of human interleukin 5 and its receptor." *Agents Actions Suppl* **46**: 23-34.

Tavernier, J., J. Van der Heyden *et al.* (2000). "Interleukin 5 regulates the isoform expression of its own receptor alpha-subunit." *Blood* **95**(5): 1600-7.

Temkin, V. and F. Levi-Schaffer (2001). "Mechanism of tumour necrosis factor alpha mediated eosinophil survival." *Cytokine* **15**(1): 20-6.

Tominaga, A., S. Takaki *et al.* (1991). "Transgenic mice expressing a B cell growth and differentiation factor gene (interleukin 5) develop eosinophilia and autoantibody production." *J Exp Med* **173**(2): 429-37.

Truant, R. and B. R. Cullen (1999). "The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals." *Mol Cell Biol* **19**(2): 1210-7.

Tu, J., N. Karasavvas *et al.* (2000). "Molecular characterization of a granulocyte macrophage-colony-stimulating factor receptor alpha subunit-associated protein, GRAP." *Blood* **96**(3): 794-9.

Umland, S. P., S. Razac *et al.* (1998). "Interleukin-5 mRNA stability in human T cells is regulated differently than interleukin-2, interleukin-3, interleukin-4, granulocyte/macrophage colony-stimulating factor, and interferon-gamma." *Am J Respir Cell Mol Biol* **18**(5): 631-42.

Umland, S. P., H. Shah *et al.* (1999). "Effects of cyclosporin A and dinactin on T-cell proliferation, interleukin-5 production, and murine pulmonary inflammation." *Am J Respir Cell Mol Biol* **20**(3): 481-92.

Valentine, J. E. and W. A. Sewell (1997). "Induction of IL-5 expression by IL-2 is resistant to the immunosuppressive agents cyclosporin A and rapamycin." *Int Immunol* **9**(7): 975-82.

van Leeuwen, B. H., M. E. Martinson *et al.* (1989). "Molecular organization of the cytokine gene cluster, involving the human IL-3, IL-4, IL-5, and GM-CSF genes, on human chromosome 5." *Blood* **73**(5): 1142-8.

Van Oosterhout, A. J., I. Van Ark *et al.* (1993). "Recombinant interleukin-5 induces in vivo airway hyperresponsiveness to histamine in guinea pigs." *Eur J Pharmacol* **236**(3): 379-83.

Van Straaten, J. F., W. H. Dokter *et al.* (1994). "The regulation of interleukin 5 and interleukin 3 gene expression in human T cells." *Cytokine* **6**(3): 229-34.

Vigneri, R., N. B. Pliam *et al.* (1978). "In vivo regulation of cell surface and intracellular insulin binding sites by insulin." *J Biol Chem* **253**(22): 8192-7.

Vodicka, M. A., D. M. Koepp *et al.* (1998). "HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection." *Genes Dev* **12**(2): 175-85.

Wahab, N. A., H. Brinkman *et al.* (2001). "Uptake and intracellular transport of the connective tissue growth factor: a potential mode of action." *Biochem J* **359**(Pt 1): 89-97.

Wakshull, E. M. and W. Wharton (1985). "Stabilized complexes of epidermal growth factor and its receptor on the cell surface stimulate RNA synthesis but not mitogenesis." *Proc Natl Acad Sci U S A* **82**(24): 8513-7.

Wan, J. (2002). *Transcriptional regulation of mouse and human interleukin-5 genes in T-lymphocytes.* JCSMR. Australian National University. Canberra

Wang, J., K. Palmer *et al.* (1998). "Circulating, but not local lung, IL-5 is required for the development of antigen-induced airways eosinophilia." *J Clin Invest* **102**(6): 1132-41.

Watanabe, S., T. Itoh *et al.* (1996). "JAK2 is essential for activation of c-fos and c-myc promoters and cell proliferation through the human granulocyte-macrophage colony-stimulating factor receptor in BA/F3 cells." *J Biol Chem* **271**(21): 12681-6.

Waters, M. J., S. W. Rowlinson *et al.* (1994). "Signal transduction by the growth hormone receptor." *Proceedings of the Society for Experimental Biology and Medicine* **206**(3): 216-20.

Waters, M. J. e. a. (2001). Growth Hormone Receptor: Nuclear Localisation. Combio, Canberra, Australia.

Watson, P. H., L. J. Fraher *et al.* (2000a). "Nuclear localization of the type 1 PTH/PTHrP receptor in rat tissues." *J Bone Miner Res* **15**(6): 1033-44.

Watson, P. H., L. J. Fraher *et al.* (2000b). "Nuclear localization of the type 1 parathyroid hormone/parathyroid hormone-related peptide receptor in MC3T3-E1 cells: association with serum-induced cell proliferation." *Bone* **26**(3): 221-5.

Weis, K., C. Dingwall *et al.* (1996). "Characterization of the nuclear protein import mechanism using Ran mutants with altered nucleotide binding specificities." *Embo J* **15**(24): 7120-8.

Welham, M. J., U. Dechert *et al.* (1994). "Interleukin (IL)-3 and granulocyte/macrophage colony-stimulating factor, but not IL-4, induce tyrosine phosphorylation, activation, and association of SHPTP2 with Grb2 and phosphatidylinositol 3'-kinase." *J Biol Chem* **269**(38): 23764-8.

Wente, S. R. and G. Blobel (1994). "NUP145 encodes a novel yeast glycine-leucine-phenylalanine-glycine (GLFG) nucleoporin required for nuclear envelope structure." *J Cell Biol* **125**(5): 955-69.

Wesche, J., A. Wiedlocha *et al.* (2000). "Externally added aFGF mutants do not require extensive unfolding for transport to the cytosol and the nucleus in NIH/3T3 cells." *Biochemistry* **39**(49): 15091-100.

Wessendorf, J. H., S. Garfinkel *et al.* (1993). "Identification of a nuclear localization sequence within the structure of the human interleukin-1 alpha precursor." *Journal of Biological Chemistry* **268**(29): 22100-4.

Wiedlocha, A., P. O. Falnes *et al.* (1994). "Dual mode of signal transduction by externally added acidic fibroblast growth factor." *Cell* **76**(6): 1039-51.

Wiedlocha, A., P. O. Falnes *et al.* (1995). "Translocation to cytosol of exogenous, CAAX-tagged acidic fibroblast growth factor." *Journal of Biological Chemistry* **270**(51): 30680-5.

Wiedlocha, A. (1996). "Retrograde transport of acidic fibroblast growth factor as a part of the growth factor signaling." *Archivum Immunologiae Et Therapiae Experimentalis* **44**(4): 201-7.

Wilson, S. J., J. K. Shute *et al.* (2000). "Localization of interleukin (IL) -4 but not IL-5 to human mast cell secretory granules by immunoelectron microscopy." *Clin Exp Allergy* **30**(4): 493-500.

Wozniak, R. W., M. P. Rout *et al.* (1998). "Karyopherins and kissing cousins." *Trends Cell Biol* **8**(5): 184-8.

Xiao, C. Y., S. Hubner *et al.* (1997). "SV40 large tumor antigen nuclear import is regulated by the double-stranded DNA-dependent protein kinase site (serine 120) flanking the nuclear localization sequence." *J Biol Chem* **272**(35): 22191-8.

Yamagata, T., K. Mitani *et al.* (1997). "Triple synergism of human T-lymphotropic virus type 1-encoded tax, GATA-binding protein, and AP-1 is required for constitutive expression of the interleukin-5 gene in adult T-cell leukemia cells." *Mol Cell Biol* **17**(8): 4272-81.

Yamaguchi, S., H. Nagai *et al.* (1994). "Time course study for antigen-induced airway hyperreactivity and the effect of soluble IL-5 receptor." *Life Sci* **54**(25): L471-5.

Yankner, B. A. and E. M. Shooter (1979). "Nerve growth factor in the nucleus: interaction with receptors on the nuclear membrane." *Proc Natl Acad Sci U S A* **76**(3): 1269-73.

Yaseen, N. R. and G. Blobel (1999a). "Two distinct classes of Ran-binding sites on the nucleoporin Nup-358." *Proc Natl Acad Sci U S A* **96**(10): 5516-21.

Yaseen, N. R. and G. Blobel (1999b). "GTP hydrolysis links initiation and termination of nuclear import on the nucleoporin nup358." *J Biol Chem* **274**(37): 26493-502.

Yasruel, Z., M. Humbert *et al.* (1997). "Membrane-bound and soluble alpha IL-5 receptor mRNA in the bronchial mucosa of atopic and nonatopic asthmatics." *Am J Respir Crit Care Med* **155**(4): 1413-8.

Yeh, H. J., G. F. Pierce *et al.* (1987). "Ultrastructural localization of a platelet-derived growth factor/v-sis-related protein(s) in cytoplasm and nucleus of simian sarcoma virus-transformed cells." *Proc Natl Acad Sci U S A* **84**(8): 2317-21.

Ylikomi, T., M. T. Bocquel *et al.* (1992). "Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors." *Embo J* **11**(10): 3681-94.

Yokoyama, N., N. Hayashi *et al.* (1995). "A giant nucleopore protein that binds Ran/TC4." *Nature* **376**(6536): 184-8.

Yoneda, Y., T. Arioka *et al.* (1987). "Synthetic peptides containing a region of SV 40 large T-antigen involved in nuclear localization direct the transport of proteins into the nucleus." *Exp Cell Res* **170**(2): 439-52.

Yoshida, M., K. Masuyama *et al.* (1996). "Local production of interleukin-5 by T lymphocytes is associated with recruitment of eosinophils in patients with eosinophilic granuloma of the soft tissue." *Int Arch Allergy Immunol* **111**(2): 133-41.

Zanders, E. D. (1994). "Interleukin-5 receptor alpha chain mRNA is down-regulated by transforming growth factor beta 1." *Eur Cytokine Netw* **5**(1): 35-42.

Zangrilli, J., N. Robertson *et al.* (2000). "Effect of IL-5, glucocorticoid, and Fas ligation on Bcl-2 homologue expression and caspase activation in circulating human eosinophils." *Clin Exp Immunol* **120**(1): 12-21.

Zasloff, M., M. Rosenberg *et al.* (1982). "Impaired nuclear transport of a human variant tRNAiMet." *Nature* **300**(5887): 81-4.

Zha, J., H. Harada *et al.* (1996). "Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L)." *Cell* **87**(4): 619-28.

Zhan, X., X. Hu *et al.* (1992). "Analysis of endogenous and exogenous nuclear translocation of fibroblast growth factor-1 in NIH 3T3 cells." *Biochem Biophys Res Commun* **188**(3): 982-91.

Zhang, D. H., L. Cohn *et al.* (1997). "Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene." *J Biol Chem* **272**(34): 21597-603.

Zolotukhin, A. S. and B. K. Felber (1999). "Nucleoporins nup98 and nup214 participate in nuclear export of human immunodeficiency virus type 1 Rev." *J Virol* **73**(1): 120-7.

Zu, X. W. and F. T. Jay (1991). "The E1 functional epitope of the human interferon gamma is a nuclear targeting signal-like element. Mapping of the E1 epitope." *J Biol Chem* **266**(10): 6023-6.